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13. ABSTRACT (Maximum 200 Words)

Genes and signaling pathways implicated in EMT and the invasiveness of breast cancers include FGF, Notch and T-box and Ets family transcription factors. One goal of this research was to examine the relationship between Tbx2/3, which is implicate din breast cancer, and EMT. Tbx2/3, was cloned and characterized. A polyclonal antibody was generated and functional assays performed to determine the role of Tbx2/3 during development and to link these observations to the tumorigenesis observed in breast cancer patients with amplifications of chromosome region 17q23 containing the Tbx2 locus. FGF signaling was also further examined during this funding period but the results of these studies were incomplete. Functional characterization of Ets showed that it is activated by the ras pathway activates and that activated Ets is involved in the specification events that are necessary for mesoderm to undergo EMT in the sea urchin.

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Introduction:

Epithelial-mesenchymal transitions (EMTs) are indicative of cancer metastasis and correlate with poor patient survival. These transitions, however, are a normal part of animal development. The EMT that occurs during mesenchyme formation in the sea urchin is highly representative of the transition that occurs during cancer metastasis in that identical signaling pathways and effector molecules are involved. This transition has been well characterized in the sea urchin and thus provides an *in vivo* model system to further elucidate the changes that cells undergo during EMTs. This study sought to identify signaling pathways and molecular correlates to EMT. In particular studies include an examination of Notch and Fibroblast Growth Factor (FGF) signaling pathways as well as members of the T-box and Ets transcription factor families. The goal of these studies is two-fold: first to enhance the knowledge on endogenous EMTs in order to have a basis of comparison for those occurring during metastasis and second, to identify new genes involved in the process and therefore provide potential targets for new therapies aimed at blocking cancer progression.

Body :

During this annual period, progress was made on the research proposed. This progress is summarized below.

Aim 1: 'What role do Ets factors play in epithelial- mesenchymal transitions'.

Ets proteins comprise a family of winged-helix transcription factors that bind to DNA monomerically at a consensus 10bp site. These factors are regulated by ras dependent MAP kinase phosphorylation of their N-terminal regulatory domains and cooperate with other ras responsive AP1 transcription factors to regulate gene expression (Yang et al., 1996; Wasylyk et al., 1997). Ras has been implicated in oncogenesis since a high number of human cancers exhibit permanently active ras molecules (Downward, 1997). Ets factors, being downstream of ras signaling, may therefore be involved in cancer progression by contributing to the transforming potential of activated ras. Additionally, Ets factors have been directly implicated in breast cancer progression and the invasiveness of certain cancers. Ets proteins overexpressed in mammary tumor cells include ESX/ESE-1, PEA3 and ERM (Dittmer and Nordheim, 1998). Invasive breast cancers produce Ets1 while non-invasive ones do not (Delannoy-Courdent et al, 1996). As well, blockage of Ets dependent transcription by either antisense Ets1 or expression of the Ets DNA binding domain alone is sufficient to block invasiveness *in vitro* (Iwasaka et al., 1996; Chen et al., 1997, Foos et al., 1998). Ets factors have been identified in the sea urchin embryo (Wei et al., 1999; Qi et al., 1992; Chen et al., 1988) and one such factor is expressed prior to an epithelial-mesenchymal transition (Kurokawa et al., 1999). Overexpression of this factor results in embryos producing an excess of mesenchymal cells, while expression of a C-terminal DNA binding domain alone exhibits the opposite phenotype. The goal of this aim was to perturb Ets function in a similar manner in order to characterize the effects on integrins, cadherins and known effectors of EMT. Another goal was to determine where Ets factors stand in the sea urchin endomesoderm gene network as well as how Ets might be regulated.

During this past period, a gene regulatory network that controls the specification of mesoderm and endoderm in the sea urchin embryo was worked out collaboratively with the laboratory of Eric Davidson at the California Institute of Technology, in conjunction with our lab. This network is based on large-scale perturbation analyses in combination with computational methodologies, genomic data, cis-regulatory analysis and molecular embryology (Davidson et al, 2002). According to the model, Ets is upstream of the EMT and appears necessary for the differentiation of the primary mesenchyme cell population. QPCR analysis places Ets upstream of known terminal mesenchymal genes, including Msp 130, Sm 50, Ficolin, Sm27, and Msp L. Additionally, Ets appears to be upstream of two more general endomesoderm specific genes, Deadringer and Fox B (Figure 1). We asked how Ets might be regulated, either by specific transcriptional activation or a phosphorylation event. A paired box transcription factor is upstream of Ets transcription (Olivieri et al, 2002, 2003). It had been shown that Ets was a downstream target of phosphorylation by the Ras-Raf-MEK-ERK signaling pathway in cell culture (Rabault et al, 1996; Fafeur et al, 1997; Paumelle et al, 2002), so an inhibitor of ERK was utilized to

determine if the pathway might work similarly in the urchin. Indeed, the inhibitor of ERK prevented the specification of mesenchymal cells and a failure of EMT. If ERK acts through Ets in this sequence, the prediction that follows is that a constituitively active form of Ets might rescue the drug-induced inhibition of EMT. This appears to be the case since the injection of RNA encoding a constituatively active Ets causes the specification of excess mesenchymal cells, even in the presence of the drug. Thus, the ras pathway, through Ets, regulates the progression of specification, leading to ingression. When Ets functions, it specifies the apparatus required for EMT. A later unknown step provides the proximal signal to initiate the EMT.

Two publications from this work were published during this period [Oliveri, P., Davidson, E.H. and McClay D.R. (2003). Activation of pmar1 controls specification of micromeres in the sea urchin embryo. *Dev Biol* **258,** 32-43; Peterson, R.E. and McClay, D.R. (2003). Primary mesenchyme cell patterning during the early stages following ingression. *Dev Biol* **254,** 68-78.] and a reprint of Oliveri et al is attached in the appendix for more detailed analysis of the results described above.

AIM 2 : 'To characterize the role of FGF and Notch signaling during epithelial-mesenchymal transitions'

Months 1-18 in the 'Statement of Work (SOW) were allocated to study Notch signaling, T-box genes, and the relation between the two. Murine mammary tumor virus (MMTV) induced tumorigenesis previously identified the INT3 gene as a locus for viral insertion (Gallahan and Callahan 1987, 1997). The INT3 gene incodes a mammalian Notch homologue, Notch4. Viral insertion generates a truncated protein consisting of only the intracellular portion of the receptor, and thus, a constitutively active signal. In the sea urchin, a similar form of the Notch protein (LvN^{act}) when expressed results in an increase in the number of migratory mesenchyme cells (Sherwood and McClay 1999).

The first aspect of this aim was to characterize a possible downstream component of the Notch pathway that may be involved in the EMT. One possible candidate, the T-box gene Brachyury, was characterized, but was shown not to be a direct target of Notch signaling. However, it was significant because the work showed that Brachyury functioned in gastrulation movements of the endoderm. Brachyury may be important in coordinating adhesive and cytoskeletal changes, since these are necessary for gastrulation movements (Gross and McClay, 2001). During the course of this study, another sea urchin homologue of a T-box gene, Tbx2, was cloned because it had recently been implicated in breast cancer progression (Barlund et al., 2000; Jacobs et al, 2000). We refer to this gene as Tbx2/3 due to the fact that the Tbx2/3 subfamily of T-box genes is represented by only one gene, which is a predecessor of the duplication event that generated individual Tbx2 and Tbx3 genes. As mentioned in the last report, Tbx2/3 expression was analyzed with polyclonal antisera generated against recombinant protein. Nuclear expression of LvTbx2/3 initiated at the mesenchyme blastula stage embryo and persisted throughout the larval stage of the embryo. Tbx2/3 was expressed asymmetrically in the dorsal region of all three germ layers of the embryo throughout this developmental time period. The molecular linkage of dorsal specific Tbx2/3 expression

within all three germ layers was demonstrated by perturbing events thought to pattern the dorsal ventral axis of the embryo. Based on these perturbations, it appears Tbx2/3 is downstream of the specification of endoderm, mesoderm and ectoderm, including the initial dorsal-ventral specification events. This data indicates that Tbx2/3 may be proximal to the morphogenic movements that shape dorsal structures.

During this past funding period, Tbx2/3 was better characterized. It appears as though early on during the initial ingression of mesenchyme cells that the expression of Tbx2/3 is dynamic. The entire population of primary mesenchyme cells expresses Tbx2/3 during their initial ingression into the blastoceol of the embryo. Later, expression is restricted to only the dorsal mesenchymal cells. This is the time that skeletal patterning begins to shape these cells into the spicule skeleton of the embryo, providing further evidence that Tbx2/3 function lies downstream of the signals that set up the dorsal-ventral axis. To determine the function of Tbx2/3, ectopic mRNA expression studies were performed. Overexpression of Tbx2/3 mRNA produced abnormal morphological phenotypes and patterning deficiencies in the derivatives of all three germ layers. Yet, normal marker expression remained in each germ layer, indicating that ectopic Tbx2/3 does not perturb germ layer specification. Therefore, the phenotype may be due to genes directly downstream from Tbx2/3 that may be involved in patterning and morphogenesis. Thus, Tbx2/3 appears to inessential for mesenchyme formation or the EMT and therefore may not be a useful target for therapeutics aimed at the EMT. Yet, the work is significant because it shows that Tbx2/3 is necessary for important morphological movements of the skeletal mesenchyme cell population which require changes in adhesive molecules and the cytoskeleton in order to occur.

Two works were published during the current period: [Gross, J.M., Peterson, R.E., Wu, S. and McClay, D.R. (2003). LvTbx2/3: a T-box family transcription factor involved in formation of the oral/aboral axis of the sea urchin embryo. *Development* **130,** 1989-1999; McClay, D.R., Gross, J. M., Range, R.C., Peterson, R.E. and Bradham, C. (2003). Sea urchin gastrulation. *Cold Spring Harbor Symp Vol* **in press**] and a reprint of Gross et al is attached in the Appendix for more detailed analysis of the results described above.

Months 18-36 of the SOW were allocated to the study of FGF signaling. Work on FGF signaling during this period has not progressed well due to the inability to isolate an endogenous sea urchin FGF receptor. However, during the past year an effort to sequence the entire genome of the sea urchins *S. purpuratus* and *L. variegatus* has progressed rapidly with support from the Human Genome Research Institute of the National Institute of Health. The sequencing is being done at the Baylor College of Medicine, Human Genome Sequencing Center, Houston, Texas. Since the inception of this effort in March of 2003, already 0.4 X of the genome has been covered. A search of this database was performed and several hits were discovered for FGF receptors. At the present time, an effort is being made to clone and characterize these receptors in order to determine if FGF signaling plays a role in the EMT of mesoderm in the sea urchin embryo.

Figure 1.

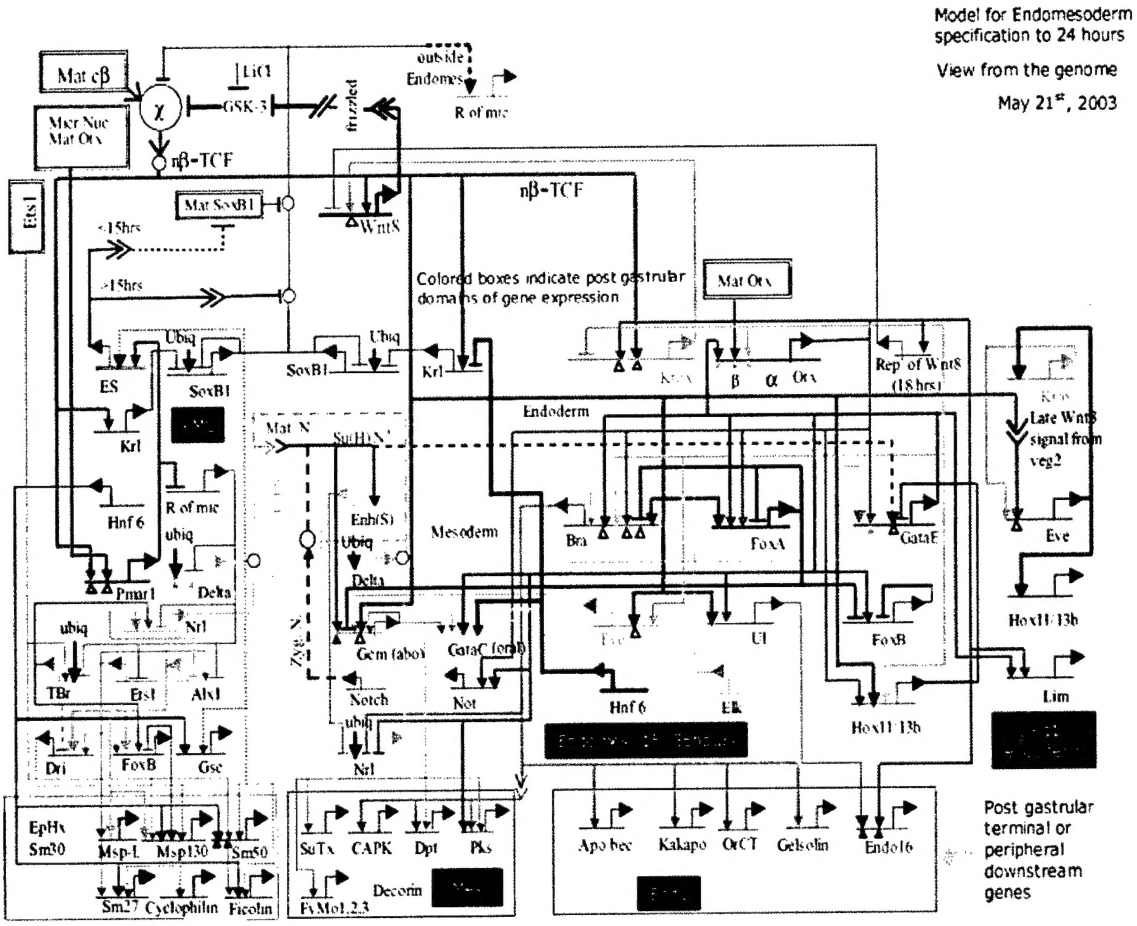
Model for Endomesoderm specification to 24 hours
View from the genome
May 21st, 2003
Mat cβ
Mat Nuc
Mat Otx
LiCl
GSK-3
frizzled
outside
Endomes
R of mic
nβ-TCF
Mat SoxB1
Wnt8
Ets1
<15hrs
>15hrs
Colored boxes indicate post gastrular domains of gene expression
Mat Otx
Ubiq
ES
SoxB1
Krl
Mat N
SuH N
Endoderm
Rep of Wnt8 (18 hrs)
Late Wnt8 signal from veg2
Hnf 6
R of mic
ubiq
Pmar1
Delta
Enh(S)
Mesoderm
Bra
FoxA
GataE
Eve
Hox11/13b
Lim
ubiq
Nrl
TBr
Ets1
Alx1
Dri
FoxB
Gsc
Gcm (abo)
GataC (otx)
Notch
Not
Hnf 6
Elk
Ui
EpHx
Sm30
Msp-L
Msp130
Sm50
Sm27
Cyclophilin
Ficolin
SuTx
CAPK
Dpt
Pks
Decorin
FxMo1,2,3
Apobec
Kakapo
OrCT
Gelsolin
Endo16
Post gastrular terminal or peripheral downstream genes

Figure 1: The endomesoderm gene regulatory network. Ets is downstream of the primary mesenchyme specific paired box transcription factor, *pmar1*. Ets is upstream of the endomeseoderm specific genes *deadringer* (Dri), and *foxb* as well as the terminal primary mesenchyme genes *msp l, msp130, sm50, ficolin and sm27.* See purple box labeled PMC.

Key Research Accomplishments:

- Characterization of the downstream targets of activated Ets.
- Characterization of the upstream phosphorylation events necessary for the activation of Ets .
- Determination that Ets functions before EMT and is necessary for the specification of mesenchymal mesoderm.
- Determination that Tbx2/3 functions downstream of dorsal-ventral specification signals, possibly as a proximal factor necessary for the expression of genes necessary for morphological changes in all three dorsal germ layers.
- Cloning of FGF receptor found in the sea urchin genome project database.

Reportable outcomes:

- Gross J.M., Peterson R.E., Wu S. and McClay D.R. (2003). LvTbx2/3: a T-box family transcription factor involved in the formation of the oral/aboral axis of the sea urchin embryo. *Development.* **130,** 1989-99.

- Poster Presentation- July 2002, 'Society For Developmental Biology Annual Meeting' Madison, WI.

Conclusions:

The overall goal of this research is to provide an *in vivo* insight into the mechanism whereby adhesions are assembled and disassembled during EMT and metastasis and thereby identify potential targets for therapeutics aimed at preventing the spread of breast cancers. Genes and signaling pathways implicated in EMT and the invasiveness of breast cancers include FGF, Notch and T-box and Ets family transcription factors. During the past funding period, studies were performed relating to each of these pathways and factors. Ets factors were shown to increase the amount of mesenchyme in the embryo and Ets was placed with the framework of the sea urchin endomesoderm network. Several genes were shown to be downstream of Ets and one upstream transcription factor was shown to impinge upon Ets transcription. It was shown that the ras pathway, through Ets, regulates the progression of specification necessary the EMT to occur. A second goal of the study was to examine the role of the T-box family member, Tbx2/3, might play in EMT. Tbx2/3 is expressed in all dorsal regions of the embryo, downstream of β-catenin or β-catenin downstream genes, BMP2/4 and likely growth factor signaling. Tbx2/3 functions in these regions in pattern formation, and possibly in the morphological movements of the mesenchyme cell population. Lastly, FGF signaling was studied. Endogenous FGF receptors were identified from the sea urchin genome sequencing database and are in the process of being cloned.

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Activation of *pmar1* controls specification of micromeres in the sea urchin embryo

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Abstract

pmar1 is a transcription factor in the paired class homeodomain family that was identified and found to be transcribed in micromeres beginning at the fourth cleavage of sea urchin development [Dev. Biol. 246 (2002), 209]. Based on in situ data, molecular perturbation studies, and QPCR data, the recently published gene regulatory network (GRN) model for endomesoderm specification [Science 295 (2002) 1669; Dev. Biol. 246 (2002), 162] places *pmar1* early in the micromere specification pathway, and upstream of two important micromere induction signals. The goal of this study was to test these three predictions of the network model. A series of embryo chimeras were produced in which *pmar1* activity was perturbed in one cell that was transplanted to control hosts. At the fourth cleavage, micromeres bearing altered *pmar1* activity were combined with a normal micromereless host embryo. If β-catenin signaling is blocked, the micromeres remain unspecified and are unable to signal to the host cells. When such β-catenin-blocked micromeres also express Pmar1, all observed micromere functions are rescued. The rescue includes expression of the primary mesenchyme cell (PMC) differentiation program, expression and execution of the Delta signal to induce secondary mesoderm cell (SMC) specification in macromere progeny, and expression of the early endomesoderm induction signal necessary for full specification of the endoderm. Additionally, Pmar1 expressed mosaically from inserted DNA constructs causes induction of ectopic Endo 16 in adjacent cells, demonstrating further that Pmar1 controls expression of the early endomesoderm induction signal. Based on these experiments, Pmar1 is an important transcription factor necessary for initiating the micromere specification program and for the expression of two inductive signals produced by micromeres. Each of the tests we describe supports the placement and function of Pmar1 in the endomesoderm GRN model.

Keywords: Gene regulatory network; Sox; Delta; β-Catenin; Paired homeodomain

Introduction

The recently published gene regulatory network (GRN) for endomesoderm specification provides a new focus for our view of early development (Davidson et al., 2002a,b). That view of development is a progression of specification sequences with many inputs, beginning with cytoplasmically localized information, and later with coordinating inputs of short-range signals from other cells. Each level of specification results from a complex integration of transcription factor inputs (Davidson, 2001). The endomesodermal GRN model integrates published fragmentary information regarding endomesoderm specification in sea urchin embryos and is further supported by additional molecules obtained in several differential screens. The many components in the network model are provisionally assigned a position based on a series of expression and perturbation studies. The value of this network is manyfold. It provides a template for further studies that will expand the details of specification. It will enable a temporal and quantitative expansion of our understanding, and importantly, it provides a template for many tests of biological functionality. The network currently is provisional since many of the nodes and positions are based on logical interpretation of experimental results. Authentication of the network requires additional research in at least two areas: biological tests of

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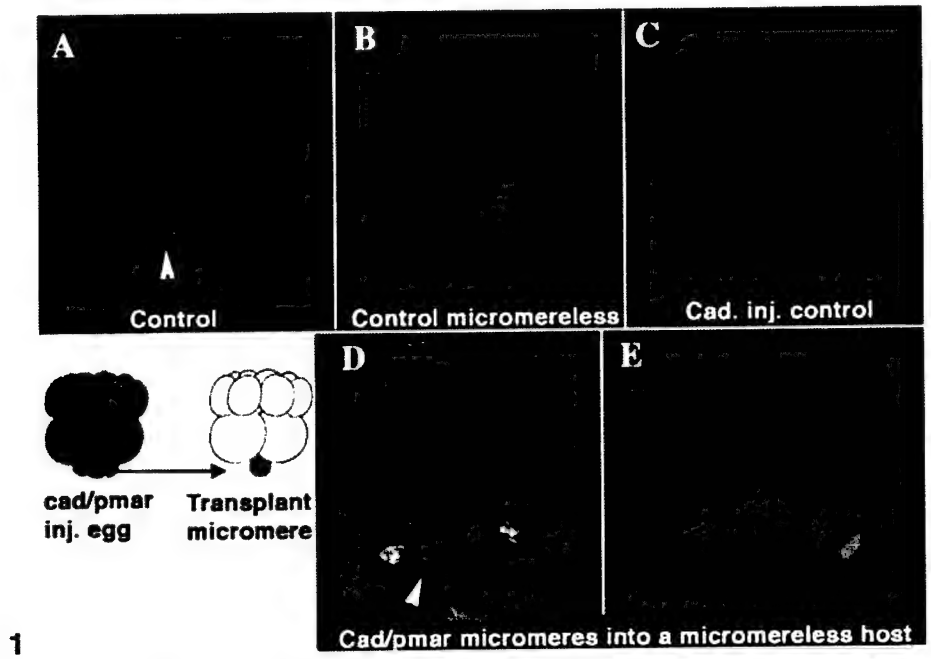
molecular function and *cis* regulatory analyses that will test and challenge predictions of the network. The present set of experiments is among the first of the biological tests that will, along with the *cis* regulatory solutions, vastly reinforce confidence in the authenticity of the network. Other efforts are underway with many genes to establish whether the predicted connections between transcription factors are direct as indicated in the current model, or whether there is a more indirect relationship between genes in question.

The specification of micromeres is a component of the larger endomesoderm GRN. This cell lineage gives rise to the PMCs, the cells responsible for production of the embryonic skeleton. Micromeres have fascinated embryologists for more than 100 years because they are so easily identified at the fourth cleavage as a result of an unequal cell division at the vegetal pole. Bovari (1901a,b), established their lineage and fate. In the 1920s and '30s, Hörstadius carried out numerous blastomere manipulation experiments, including micromere transplantation (reviewed by Hörstadius, 1939). Micromeres transplanted to the animal pole of a host 16-cell-stage embryo induced an ectopic gut that invaginated from the site of transplantation. These results suggested that micromeres somehow induce endoderm and are important for placement of axial properties. In the absence of micromeres, the embryo lacks a gut (after a long delay, the embryo sometimes regulatively compensates and eventually replaces the gut). Ransick and Davidson (1993) later showed that micromeres begin endoderm induction very early, and that the induction of an ectopic gut at the animal pole exhibits expression of the same molecular markers as the endogenous gut. These experiments suggested that micromeres begin their specification sequence immediately after fourth cleavage and that micromeres are an important source of an early inductive signal (ES) for the endomesoderm lineage.

Embryological experiments demonstrated that micromeres themselves require no further input from the rest of the embryo beyond fourth cleavage since micromeres, isolated at the 16-cell stage, differentiate as PMCs and make skeletal spicules in culture (Okazaki, 1975). These cells require horse serum, so a caveat of the above experiment is the potential requirement for growth factors or other factors present in the horse serum. Nonetheless, numerous experiments have shown that micromeres isolated at fourth cleavage and transplanted to host embryos give rise exclusively to skeletogenic progeny, no matter where they are placed, so by fourth cleavage, in the context of the living embryo, they display autonomous specification. At the 16-cell stage, the nuclei of micromeres become positive for β-catenin, a transcriptional cofactor in the wnt signaling pathway (Logan et al., 1999; Peifer et al., 1991), and β-catenin is necessary for micromere specification (Emily-Fenouil et al., 1998; Logan et al., 1999; Wikramanayake et al., 1998). Though β-catenin is the earliest known input in micromere specification, other components of micromeres have been found to participate in steps that lead to PMCs. Several transcription factors are expressed exclusively in the micromeres, and perturbation of these has substantial effects on PMCs (Kurokawa, 1999; Davidson et al., 2002b; Amore et al., 2002). The endomesoderm GRN models a hierarchical pattern of gene activation and repression based on perturbation of one and observing the effect on expression of other transcription factors known to be in the micromeres. Based on logic, these studies reveal that maternal nuclear β-catenin and Otx activate transcription of *pmar1* at fourth cleavage, and Pmar1 then initiates the micromere specification sequence. *pmar1* is proposed to be a direct target for the β-catenin, and is further proposed to be upstream of the early endoderm induction signal (ES), the expression of Delta, and the transcriptional apparatus that specifies the differentiated PMCs. Several substantial pieces of information lead to this model. First, Pmar1 is expressed specifically in micromeres beginning at the fourth cleavage, so it is expressed in the right place at the right time. Second, experimental activation of *pmar1* in other parts of the embryo causes an ectopic specification of mesenchyme-like cells. Third, upregulation of *pmar1* leads to upregulation of other micromere lineage transcription factors, but the reciprocal activation is not true (Oliveri et al., 2002).

A later functional property of micromeres is the expression of the Delta ligand to activate Notch in veg 2 cells, a necessary step in the induction of SMCs (Sherwood and McClay, 1999). Delta is released between the 8th and 10th cleavage and is expressed by micromeres at the right time for this signal to activate Notch on the adjacent veg 2 cells (Sweet et al., 2002). Thus, shortly after the micromeres initiate their own specification, they begin to signal to other lineages in the embryo. Ransick and Davidson (1993) showed that a different micromere signal induces endoderm beginning between the 4th and 6th cleavage.

Given this rich background, there are two sources of information necessary to validate the GRN model for the micromeres and extend it. First, since many of the perturbation studies utilize injections that alter gene regulation in the entire embryo, biological studies are necessary to authenticate the function of the GRN in the specific cells where specification events are thought to occur. Second, *cis* regulatory analysis of *pmar1* and other genes in the micromere GRN will strengthen confidence in the proposed transcriptional activation sequence. The purpose of this paper is to test experimentally the micromere portions of the GRN, especially as they relate to *pmar1* function, using blastomere transplantation approaches. Experiments show that all three major functions of the micromere progeny are dependent on *pmar1* function. The ES and the Delta signal are downstream and essentially independent of the regulatory apparatus controlling PMC differentiation. Thus, we confirm that *pmar1* activation is one of the most crucial steps in the activation and specification of the micromere lineage.



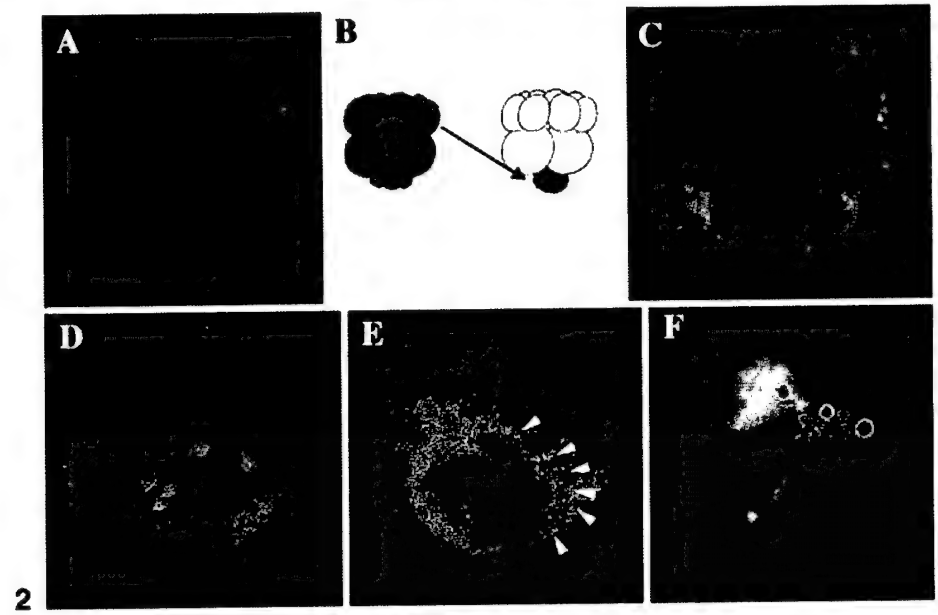


Fig. 1. Pmar1 rescues embryos in which β-catenin nuclearization is prevented. All embryos at 36 h. **(A)** Control embryo. PMCs are stained with a PMC marker, and in the center of the PMC ring, the blastopore of the archenteron is visible (arrowhead). **(B)** An embryo as in (A) but with micromeres removed at the 16-cell stage. The embryo has no PMCs, and no archenteron (16/16 cases). **(C)** Micromeres from embryos injected with Δ-*cadherin* were returned to a micromereless embryo as in the inserted diagram, grown to 36 h, then stained with the PMC marker. There were no PMCs and no archenterons (35/36 cases). Control micromeres added to micromereless embryos resembled control embryos with PMC rings and archenterons (17/17 cases; data not shown). **(D, E)** Micromeres from embryos injected with both Δ-*cadherin* RNA and *pmar1* RNA were transplanted to the vegetal pole of micromereless control embryos. The embryos had PMC rings and archenterons (arrowhead in D) (29/30 cases). Green stain is a PMC marker; red stain (in D) or blue stain (in A–C and E) is directed against β- catenin in adherens junctions. In all experimental cases, one micromere was transplanted to the vegetal pole of a micromereless host.

Fig. 2. Pmar1 expression is sufficient to specify any blastomere as a skeletogenic mesenchyme cell precursor. (A) Host micromereless embryos that do not receive micromeres at the vegetal plate have no PMCs and fail to gastrulate by 38 h (18/18 cases). (B) The experiment. Eggs were injected with Δ-*cadherin* RNA, *pmar1* RNA, and rhodamine dextran. At the 16-cell stage a single, double-injected mesomere (red cell in diagram) was transferred to the vegetal pole of a micromereless control embryo. (C) The Pmar1-expressing mesomeres ingress as PMCs, stain with a PMC marker (red), and the archenteron is normally completed by 38 h (17/18 cases). (D) Ingressed mesomeres (stained green with a PMC marker) form a normal-looking PMC ring. The red stain is β- catenin in adherens junctions. (E) When transplanted to the vegetal plate of embryos with endogenous micromeres, the double-injected mesomeres still make PMCs, (arrowheads pointing to red-stained mesomere-neo-PMC cells) and join into the vegetal plate ring along with the endogenous PMCs. (F) The skeleton produced by a Pmar1- expressing mesomere transformed into a PMC is entirely of mesomere origin (rhodamine-labeled mesomere cells are pseudocolored in the host embryo, and an additional spicule is out of focus in the background).

Materials and methods

Micromanipulation and imaging

Eggs of *Strongylocentrotus purpuratus* were shed in SW following injection of animals with 0.5 M KCl. Eggs were washed in SW and fertilized with dilute sperm in SW containing para-aminobenzoic acid (15 mg/100 ml SW). Cultures were incubated at 16°C. Just prior to surgery, the embryos were passed through 102-micron Nitex mesh four times to remove fertilization envelopes. Alternatively, if embryos were previously injected with RNA, they were pipeted into a narrow bore pipet that manually shears off the fertilization envelope without damaging the embryo. Surgeries were adapted from McClay et al. (2000). Briefly, to transplant cells from *S. purpuratus,* it was necessary to pretreat the embryos by incubation for 30 s in HEM (McClay, 1986), containing 2 mg/ml BSA (HEM/BSA). The BSA eliminates problems with the very sticky hyaline layer surrounding the *S. purpuratus* embryo, and the HEM softens the hyaline layer so that cells can be transplanted.

Embryos to be manipulated were transferred from the HEM/BSA to modified Kiehart chambers (Kiehart, 1982). The chambers were modified so that two glass needles could be inserted into the chamber at 90° to each other. One of the glass needles served as a suction pipet, and the other needle served as a holding and manipulating pipet. These were driven by two joystick micromanipulators. The suction applied was by mouth. For cell transplantations, donor cells were pulled into the suction pipet one at a time. The bore of the pipet was preadapted to a slightly smaller I.D. than the diameter of the cell to be transplanted so that inside the pipet the cell took on a sausage shape. The cell was transferred to a host embryo and inserted into position. The sausage shape helped with the insertion between cells in that the donor cell overlapped initially both to the inside and the outside of the host cell layer. Within a minute, the donor cell rounded up and was retained in position by the host. Embryos were then transferred back to SW. For all operations, the embryos were in HEM for less than 5 min each, and in sham control cell to control embryo operations, development was indistinguishable from unoperated control embryos both in timing of morphogenetic events and in final phenotype.

Several operations were performed. Many of the experiments required that host embryos were first made micromereless. Micromeres were removed early in the 16-cell stage and the embryos were inspected to assure that all 4 micromeres were removed. Donor cells were inserted in the cleft between the 4 macromeres. When these embryos were inspected at the 32-cell stage, the transplanted donor cells were in the same position as control micromeres relative to macromeres. Because the insertion cleft is small, generally only 1 or 2 of the 4 micromeres were transplanted. Nevertheless, previous work demonstrated that 1 micromere conveys an inductive signal that is virtually indistinguishable compared with the normal 4 micromeres (with only a reduction in the number of pigment cells indicating a reduced level of induction) (McClay et al., 2000).

When mesomeres were transplanted to the vegetal or animal poles, they were taken from the donor embryo at the 32-cell stage and 1 mesomere daughter was transplanted into the same position as micromeres. When mesomeres (or micromeres) were transplanted to the animal pole, they were inserted between the 4 blastomeres closest to the animal pole, again inserting the sausage-shaped cell into the cleft. The transplanted donor cells were prelabeled with rhodamine dextran (10,000 mw; 1:5 dilution of a 50-mg/ml stock into the injection medium). We avoided the lysinated form of rhodamine dextran, which prevented the dye from being fixed with the tissue; in preliminary experiments, the high lysine content of the fixable dye tended to reduce the effectiveness of injected molecules.

Prior to fixation, the embryos were examined by fluorescent microscopy and scored for phenotype. They were then fixed for 20 min with 2% paraformaldehyde in SW, postfixed 5 min in MeOH (on ice), and washed into SW containing 4% normal goat serum (SW-NGS). Throughout antibody-staining reactions, the embryos remained in SW-NGS and all antibodies were diluted in this solution. Embryos were stained with antibodies to PMCs [1D5, a monoclonal recognizing MSP-130 (Wessel and McClay, 1985)], β-catenin (Miller and McClay, 1997a), cadherin (Miller and McClay, 1997b), SoxB1 (Kenny et al., 1999), pigment cells (Gibson and Burke, 1985), and Notch (Sherwood and McClay, 1997). Embryos were examined by confocal microscopy using either a Zeiss 410 or 510 scanning confocal microscope.

DNA and RNA constructs and injection of into eggs

A Hatching Enzyme promoter–*pmar1* DNA construct was engineered by replacing the GFP coding region already present in a HE-GFP clone described by Bogarad et al. (1998). The GFP was removed by using the *Nhe*I and *Xho*I unique sites present in the construct, and the coding region of *pmar1,* obtained by digestion with *Spe*I and *Xho*I, was inserted (Oliveri et al 2002). The subclone was checked by restriction digestion analysis of known sites and by sequencing. The DNA clone obtained was linearized by using *Kpn*I and injected as described by McMahon et al. (1985), with the exception of the glycerol in the injection solution.

RNAs for injection were synthesized as described by Oliveri et al. (2002). For micromere rescue and blastomere micromanipulation experiments, RNA was injected along with rhodamine dextran (10 pg/pl; Sigma). The injection concentration of the probes was: Δ-*cadherin* RNA (60 ng/μl), *pmar1* RNA (5.6 ng/μl) for both the single injection and the coinjection experiments. The injection volume for each egg was approximately 2 pl. In experiments to determine the minimum amount of *pmar1* sufficient to rescue the

Δ-*cadherin* phenotype, Δ-*cadherin* RNA at 60 ng/μl was coinjected with *pmar1* RNA at 5.6, 2.8, 1.1, and 0.56 ng/μl.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described by Ransick and Davidson (1995), with the following modifications. The *endo16* probe was transcribed in presence of 10× DIG RNA labeling mix (Roche) and the GFP probe in presence of 10× Fluorescein RNA labeling mix (Roche). The two probes were hybridized to fixed embryos at the same time under the conditions described by Oliveri et al. (2002). The detection of the two different probes was obtained following the conditions described by Hauptmann (2001) and using the NBT/BCIP ready-to-use tablet (Roche) for the DIG-labeled probe and the Fast Red (Roche) for the Fluorescein-labeled probe.

Results

Pmar1 expression rescues cadherin-inhibited specification of micromeres

β-Catenin enters the nuclei of micromeres shortly after their birth at the fourth cleavage (Logan et al., 1999). Experimentally, the nuclear entry of β-catenin and its subsequent association with TCF-Lef (Vonica et al., 2000) is required for micromere specification. If nuclear entry of β-catenin is blocked by expression of the cytoplasmic tail of cadherin (Wikramanayake et al., 1998), or by blocking GSK-3 (Emily-Fenouil et al., 1998), micromeres fail to be specified. Oliveri et al. (2002) found that one of the first markers to be expressed in micromeres following nuclear entry of β-catenin is *pmar1*, a transcription factor of the paired homeodomain family. *pmar1* expression is greatly diminished if β-catenin fails to enter the micromere nuclei. Manipulation of *pmar1* in whole embryos demonstrated that it acts as a repressor. The most direct evidence for this is that introduction of mRNA encoding an Engrailed (en) repressor domain with *pmar1* homeodomain fusion forces the Pmar1-en fusion to act as an obligate repressor for target genes. This construct produces identical effects to introduction of mRNA encoding the natural Pmar1 protein. Both cause all downstream micromere genes to be globally derepressed. Therefore, the micromere-specific expression of the *pmar1* gene normally has the effect of repressing a gene encoding a repressor of these downstream genes. It thereby allows them to become active exclusively in micromeres and their descendants. In addition, the Pmar1 protein includes a sequence motif that shows similarities to several well-known transcriptional repressors (Oliveri et al., 2002).

To ask how pmar1 works in the micromeres, we began a series of studies in which expression of *pmar1* was modified only in the micromeres. Three functions were examined: (1) expression of the ES (Ransick and Davidson, 1995), a signal necessary for correct specification of the endomesoderm and for on-time invagination of the archenteron, (2) expression of the Delta signal, necessary to activate Notch in the veg 2 cells to specify SMCs (Sherwood and McClay, 1999; Sweet et al., 2002); and (3) differentiation of micromere progeny into skeletogenic mesenchyme cells. Earlier transplantation studies showed that, if normal micromeres are transplanted from a donor 16-cell-stage embryo to a host micromereless embryo, the micromeres behave just as they do in an unmanipulated embryo (McClay et al., 2000). If a micromereless host received transplanted micromeres, the micromeres ingress as PMCs, express surface molecular markers, and produce a skeleton. The embryo gastrulates at the correct time (32–36 h for *S. purpuratus* at 16°C), SMCs are specified normally, based on the appearance of pigment cells by 48 h, then coelomic pouches, a muscular pharynx, and blastocoelar cells in pluteus larvae at 72 h. If, in the same operation, micromeres are not replaced, as in Fig. 1B, most embryos fail to gastrulate. In a large number of trials, many fewer than 20% of the micromereless embryos eventually gastrulate. Those few embryos that eventually recover and gastrulate become albino pluteus larvae, their archenteron invagination is delayed to beginning at 55–72 h, and PMCs plus nonpigmented SMCs become replaced only in those embryos that eventually gastrulate. Thus, gastrulation and specification of SMCs are severely compromised by the absence of micromeres. In embryo recombinations, when β-catenin-induced input was eliminated from micromeres by transplantation of Δ-cadherin-expressing micromeres to replace normal micromeres on uninjected host embryos, the embryos developed as if they were micromereless (Fig. 1C). The chimeras lacked PMCs, invagination of the archenteron was greatly delayed if it occurred at all, and all such embryos were albino, presumably as a consequence of a failure of the micromeres to express Delta.

When *pmar1* RNA was injected along with the same β-catenin-inhibiting concentration of Δ-cadherin, and micromeres transplanted at the 16-cell stage, normal- appearing development returned (Fig. 1D and E). The double-injected micromeres became PMCs, a skeleton formed, and archenterons invaginated at the correct time (32- 36 h), in almost all of the transplant combinations (greater than 95% of 136 transplant combinations in 19 separate experiments) (Fig. 1D and E). When embryos were cultured longer, they displayed the full range of SMCs, including pigment cells (see Fig. 3 below), a muscular pharynx, coelomic pouches, and blastocoelar cells. Thus, expression of *pmar1* rescued all the known functions of micromeres, including production of both induction signals and the production of a normal skeleton. Whole embryos that expressed *pmar1* alone or Δ-cadherin plus *pmar1* formed a mass of mesenchymal cells.

The Pmar1 rescue of micromeres is effected by fewer copies of RNA than normally are synthesized by micromeres

A general concern of the RNA injection experiments, like the rescue experiment described above, is that the phenotype observed could be caused by a vast overexpression of *pmar1* that forces an artifactual conversion of cells to the mesenchyme phenotype. Although the Δ-*cadherin: pmar1* ratio of injected mRNA was 10:1, the described rescue of the β-catenin-devoid phenotype was obtained with the injection of roughly 20- to 40-fold excess amount of *pmar1* mRNA into whole eggs, relative to calculated amounts of endogenous *pmar1* RNA in micromeres (see Materials and methods for injection conditions). If one conservatively considers no degradation of the injected RNA and assumes equal partitioning of the injected RNA throughout the cytoplasm, the micromeres at the moment they are born (4th division) will inherit just 1.6- to 3.2-fold excess *pmar1* mRNA relative to untreated embryos [the micromeres segregate 8% of egg cytoplasm (Davidson, 1988)]. Thus, the observed rescue is unlikely to result from vast overexpression of *pmar1*.

To experimentally establish the minimum amount of *pmar1* necessary to rescue micromere specification, we analyzed effects of a dilution series of RNA injections. One might predict that the dominant repressor function of Pmar1, to extinguish a ubiquitous repressor and thereby activate the micromere-PMC cell program, can be reached with a relatively low number of transcripts. To test that hypothesis, we determined the smallest amount of *pmar1* necessary to overcome the Δ-cadherin-altered phenotype. *pmar1* RNA at increasing dilutions was injected into eggs along with the minimum number of molecules of Δ-*cadherin* RNA known to eliminate β-catenin signaling (Δ-*cadherin: pmar1* ratio of transcripts 20:1, 40:1, and 100:1). Embryos were scored at 24 h of development for the resulting phenotype. Using this approach, we find the minimum number of *pmar1* copies/cell necessary to rescue the micromere specification, as well as transfate the other blastomeres, is 0.4- to 0.8-fold the normal amount of *pmar1* RNA present in micromeres at 5th cleavage. This corresponds to about 66–130 copies per micromere relative to 163 copies/cell maximally present in normal embryos between the 4th and 8th cleavage (Oliveri et al., 2002). Thus, *pmar1* rescues micromere specification even when present at a fraction of levels expressed in control embryos.

Ectopic Pmar1 expression specifies the micromere program in all cells of the embryo

In micromeres, the endomesoderm GRN predicts Pmar1 to repress a ubiquitous repressor expressed everywhere in the early embryo. If this ubiquitous repressor normally blocks micromere specification elsewhere in the embryo as well, the model predicts that ectopic expression of *pmar1* should force nonmicromeres into the micromere specification pathway. The mesenchymal phenotype of *pmar1* mRNA-injected whole embryos tends to support this hypothesis. But, are those Pmar1-expressing cells converted to functional micromeres? To address this question, *pmar1* RNA was either injected with Δ-*cadherin* RNA or alone (with an identical phenotypic outcome). At the 16-cell stage, a single *meso*mere (or macromere in other cases) was removed from the animal pole of injected embryos and transplanted to the vegetal pole in place of four removed micromeres of an uninjected control embryo. Mesomeres normally produce only oral and aboral ectoderm. Fig. 2 shows the remarkable result that the progeny of the transplanted Pmar1-expressing mesomeres behave in almost every way like micromeres. The Neo-PMCs of the chimera ingress, the archenteron invaginates at the correct time demonstrating that the transplanted mesomere conveys the ES (Fig. 2C). The transformed mesomeres make a normal-appearing PMC ring (Fig. 2D and E), and they make a skeleton (Fig. 2F) (18/18 cases). When examined at 48 h., embryos with transformed mesomeres are pigmented (Fig. 3C), suggesting that the Delta ligand was produced correctly (4/6 cases), while control micromereless embryos (18/18), micromereless embryos that received uninjected mesomeres (9/9), or micromereless embryos that received mesomeres with Δ-*cadherin* only (6/6), failed to gastrulate, failed to make PMCs, and failed to make pigment cells (data not shown). Pluteus larvae with Pmar1-tranformed mesomeres were observed to have a muscular pharynx, coelomic pouches, and blastocoelar cells, indicating that the full range of SMCs was specified (data not shown). The only abnormal aspect of the mesomere-PMC conversion observed was the somewhat abnormal pattern of the skeleton. With this exception, early ectopic *pmar1* expression is able to convert a mesomere from its normal ectodermal fate into cells of the micromere-PMC lineage, and the conversion is almost complete. A similar outcome is observed if *pmar1*-injected macromeres are transplanted to the vegetal plate (data not shown). We conclude that expression of *pmar1* is sufficient to launch the micromere specification program. Since any cell expressing Pmar1 is specified as a micromere/PMC, we suggest that no other maternal components are necessary in micromeres other than those that activate *pmar1*.

Pmar1 expression in micromeres is upstream of the two signals that induce endoderm and secondary mesoderm

The SMC induction signal, Delta, is expressed by micromeres between the 8th and 10th cleavage (McClay et al., 2000; Sherwood and McClay, 1999; Sweet et al., 1999 2002). Previous work showed that ectopic *pmar1* expression activates ectopic *delta* RNA expression (Oliveri et al., 2002). To confirm that Pmar1 is upstream of Delta, and importantly, that the Delta produced in response to *pmar1* activation has SMC inductive activity, we manipulated *pmar1* in micromeres and mesomeres as above and then

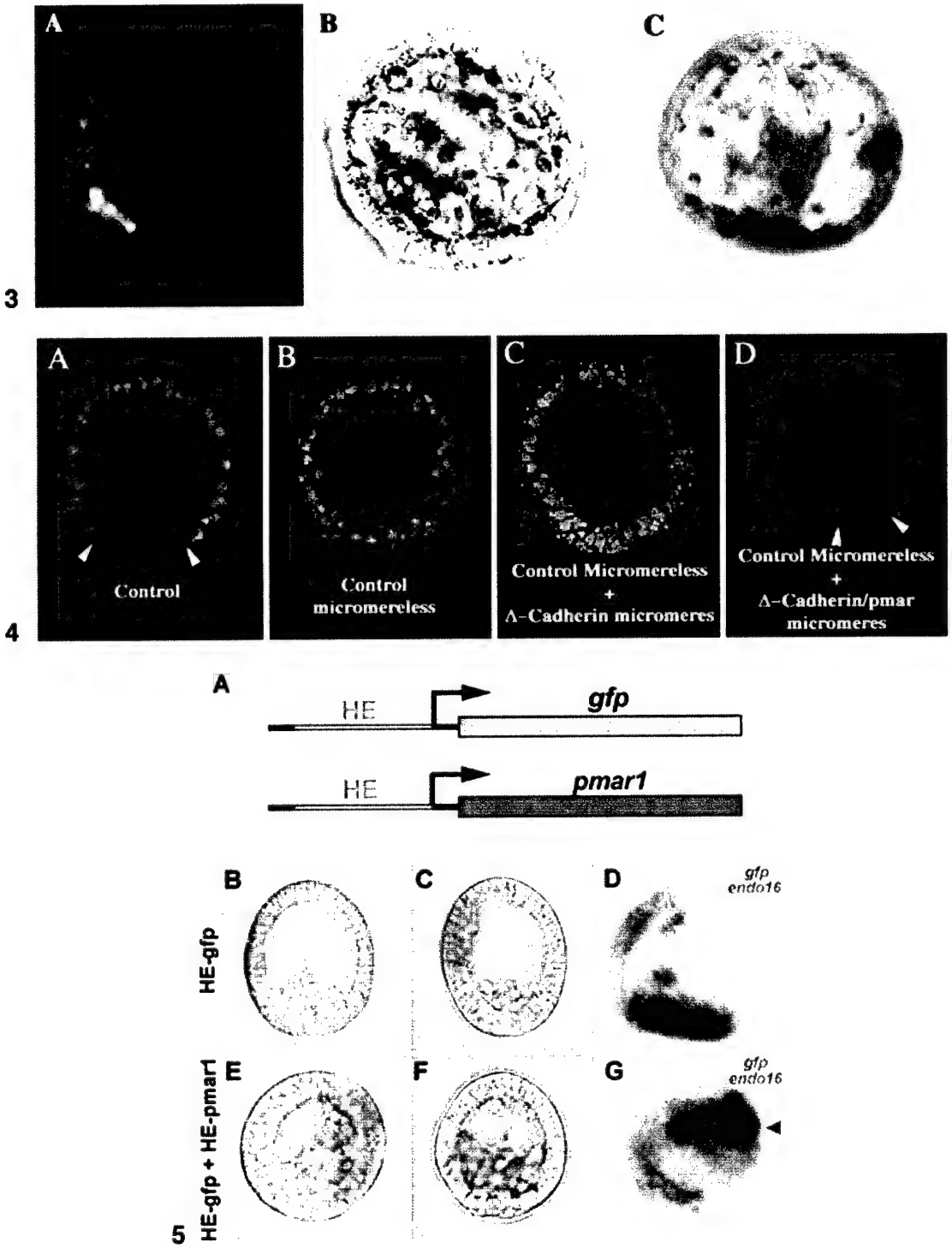


Fig. 3. Pmar1 expression is necessary for production of the SMC induction signal. (A, B) *cad/pmar1* double-injected micromeres were transplanted to the vegetal pole of control micromereless host embryos. (A) At 48 h, the *cad/pmar1*-injected cells were PMCs (Portion of ring of fluorescent cells in A). (B) At the same plane of focus, that embryo also had nonfluorescent (i.e., induced) pigment cells (arrowheads) (5/6 cases examined). (A) Epifluorescent image of the same embryo showed in (B) (bright field). (C) *pmar1*-expressing mesomeres transplanted to the vegetal pole of micromereless embryos also induce the host to produce pigmented cells (arrowheads) (4/6 cases examined). Control micromereless embryos were albino (6/6 cases examined in this experiment; data not shown).

Fig. 4. *Pmar1* is upstream of the early endoderm induction signal. An assay for a consequence of the ES signal examines nuclear SoxB1 immunocytologically (green nuclei). In each case, PMCs were removed as micromeres or ingressed, leaving only endomesoderm at the vegetal plate (with the exception of C).

examined the ability of those micromeres, when transplanted to the vegetal plates of unmanipulated micromere-less embryos, to induce pigment cells. Fig. 3 shows that when Pmar1 is present in the transplanted micromeres, a pigmented embryo results by 48 h. (23/30 cases). Embryos are albino in the absence of *pmar1*-expressing micromeres (6/6 analyzed cases). Manipulation of *pmar1* in micromeres has the predictable effect on that induction sequence (no *pmar1* expression, no induction). Embryos with *pmar1*-expressing mesomeres also produce pigment cells by 48 h (Fig. 3C). If grown to the pluteus stage, embryos with transplanted Pmar1-expressing micromeres or mesomeres also have a muscular pharynx, coelomic pouches, and blastocoelar cells (data not shown). We conclude that *pmar1* expression is sufficient for the production of the Delta induction signal, a signal that is necessary for SMC specification.

The early endomesoderm induction signal (ES) is first detected between the fourth and sixth cleavage (Ransick and Davidson, 1995), i.e., if micromeres are present during that time *endo 16* message is later detected normally in the vegetal plate. If micromeres are removed, *endo 16* expression is reduced progressively depending on whether micromeres are removed at the 6th, 5th, or 4th cleavage. The provisional endomesoderm GRN proposes *pmar1* expression to be required for rapid activation of the ES in the micromeres (Davidson et al., 2002a,b). The next tests examined this prediction that place *pmar1* immediately upstream of the production of the ES.

To examine the ES, two assays were developed that require this signal. The first assay measured disappearance of nuclear SoxB1 (Fig. 4). SoxB1 is a transcription factor present maternally in all cells (Kenny et al., 1999). Early in development, maternal SoxB1 protein concentration is greatly reduced in micromere nuclei, and after hatching is cleared from the nuclei of veg 2 cells and ultimately to the entire endomesoderm region. Fig. 4 shows that SoxB1 clearance requires the presence of micromeres and that requirement fits parameters of the ES. An assay was designed, using immunochemical disappearance of nuclear SoxB1 from the veg 2 cells as a measure, to examine the proximal consequences of the ES (Fig. 4). The ES also is a prerequisite for on-time gastrulation and for later expression of *endo 16* in the vegetal territory, providing two additional another assays to assess ES activity (Ransick and Davidson, 1995) (Fig. 5). We used both of these assays to ask if *pmar1* expression in cells is necessary for the ES response in neighboring cells.

If micromeres are absent, there is no SoxB1 clearance from the vegetal plate (Fig. 4B) at 20 h (early mesenchyme blastula stage), and the archenteron fails to invaginate until at least 24–36 h beyond normal invagination time in a small percentage of embryos. Control embryos with micromeres eliminate SoxB1 from a large region of the vegetal plate that is about 13 cells wide, as counted from confocal sections (Fig. 4A), and gastrulate beginning at 30–32 h. If Δ-*cadherin*-expressing micromeres are transplanted to micromere-less control embryos, SoxB1 clearance from endomesoderm fails to occur (Fig. 4C) and the archenteron fails to invaginate. If the transplanted micromere expresses a rescuing level of Pmar1, SoxB1 is cleared from the veg 2 cells, indicating that the ES is produced and functional (Fig. 4D), and the archenteron begins invagination at 30–32 h.

Fig. 5 demonstrates that *pmar1* is upstream of the early signal using a different experimental approach. The entire coding region of *pmar1* was placed under the control of the hatching enzyme (*HE*) *cis* regulatory element. The *HE* promoter is a well- characterized regulatory region that allows expression of a downstream gene everywhere in the embryo except in the most vegetal territories (Wei et al., 1997). Groups of 200– 300 eggs were injected with constructs: hatching enzyme promoter-driven *pmar1* (HE- *pmar1*) and/or the hatching enzyme promoter-driven *gfp* (*HE-gfp*) (Bogarad et al., 1998). Double injected embryos concatenate the two DNA constructs and express them mosaically, in the same cell(s) (Arnone et al., 1997). Thus, if double injected, a *gfp*- expressing clone of cells will also express *pmar1*. The easily detected GFP therefore is used as an ectopic *pmar1* expression marker. As shown already by the whole embryo mRNA injections, all cells of the embryo have the capability of acquiring a micromere fate if they express *pmar1* (Oliveri et al., 2002). Therefore, ectopic cell

(A) Normal SoxB1 clearance from the endomesoderm cells of the vegetal plate by 20 h (316/316 controls examined in 19 experiments). (B) If micromeres are removed during 4th cleavage, SoxB1 fails to clear from the endomesodermal vegetal plate (34/36 cases examined). (C) If micromeres lacking β-catenin input are present at the vegetal plate, those micromeres fail to convey the ES, and no SoxB1 is cleared from vegetal plate nuclei (3/3 cases examined) (D) Pmar1 expression by transplanted micromeres rescues the ES as seen by the vegetal clearance of SoxB1 from endomesoderm. The Pmar1-expressing PMCs ingressed (as they did in controls) (11/12 cases examined). All embryos shown were fixed at 20 h of development. Micromeres were labeled with rhodamine dextran to track location of micromere progeny. Prior to fixation, the location of PMCs was noted; then, with fixation, the red dye was lost from the cells.
Fig. 5. Mosaic expression of *pmar1* in ectopic positions leads to endoderm induction in adjacent cells. (A) Schematic map of the two constructs used to induce mosaic ectopic *pmar1* and *gfp* expression via DNA injection. Both the *pmar1* coding sequence (red) and the *gfp* sequence (green) were placed under the control of the hatching enzyme promoter (HE). The hatching enzyme regulatory sequence normally drives expression ubiquitously, except in micromeres. (B, C) Embryos injected only with *HE-gfp* construct. As expected, GFP was expressed mosaically in patches of cells, except by PMCs as indicated by the green false color, in 95% of the cases. (E, F) Eggs received *HE-gfp* and *HE-pmar1* DNA. Again, GFP was expressed in a mosaic pattern (green cell clones), but in these embryos, GFP was expressed abundantly in PMCs, indicating that, wherever *pmar1* is expressed, the cells were transformed into PMCs. Note that the embryos show extra PMCs and a reduced blastocoel. In (D), eggs were injected with the *HE-gfp* construct and were examined by double in situ analysis at mesenchyme blastula stage with probes to *gfp* (brown-red) and an endoderm marker, *endo16* (blue-purple). The *gfp* is expressed only in non-PMC tissues, and *endo16*, as expected, is expressed in the vegetal plate only. By contrast, in (G), eggs that had been double injected with the *gfp* and *pmar1* constructs express *endo16* (blue-purple) in an extra patch of cells (purple arrowhead) adjacent to *gfp* (and *pmar1*)-expressing cells ingressed in the blastocoel.

clones expressing *pmar1* should exhibit micromere functions, including release of induction signals. Fig. 5B and C shows that, when the GFP reporter is expressed alone, it is expressed mosaically in *non*micromeric regions in all of the analyzed cases, as expected. The HE promoter drives the expression of the GFP either in single clones (Fig. 5C) or in multiple different clones (Fig. 5B) in 85–99% of the injected embryos, depending on the embryo batch. By contrast, at 24 h of development, GFP-expressing cells in the double injected embryos ingress into the blastocoel reflecting the behavior of PMCs (Fig. 5E and F). These embryos, in 86% of the cases, show reduced blastocoelar cavities with extra mesenchyme cells in them. Thus, the HE promoter activates *pmar1* expression ectopically. This activates the micromere-PMC regulatory program, and as a consequence, GFP now is expressed in mesenchyme cells.

The next test asked if those double-injected, transformed cells convey the early induction signal. Ectopic expression of *pmar1* not only should convert cells into the micromere lineage as described above, but also should induce adjacent cells to be specified as endomesoderm. This prediction was tested by double in situ detection of *endo16,* an early endomesoderm marker, and *gfp* at 24 h of development after coinjection of *HE-gfp* and *HE-pmar1* (on 66 embryos) or *HE-gfp* alone as control (on 58 embryos). In Fig. 5G, the *gfp/pmar1* expression (red-brown) is shown to be in cells that have ingressed into the blastocoel from an ectopic position relative to the endogenous vegetal plate (*endo16* stain at the bottom of Fig. 5G). This phenotype is observed in 88% of the injected embryos. Embryo detection of RNA by in situ hybridization was about the same as direct observation of the GFP (88 vs 86% of embryos expressing extra mesenchyme cells). Adjacent to the ectopic *gfp/pmar1* cells, ectopic *endo16* expression (dark blue-purple) is seen in an ectopic patch of cells (arrowhead in Fig. 5G), with the endogenous patch of *endo16* seen at the bottom (75% of the scored double injected embryos show adjacent patches of cells positive for *endo16* expression). Embryos injected with *HE-gfp* alone show the normal vegetal plate expression of *endo16* and nonmesenchyme patches of *gfp*-expressing cells in 98% of the cases (Fig. 5F). We conclude from these two experiments that *pmar1* is upstream of the ES.

Pmar1 expression is upstream of secondary endomesoderm induction

In the classic Hörstadius experiment, a second gut is induced if micromeres are transplanted to the animal pole of the sea urchin embryo (Hörstadius, 1939; Ransick and Davidson, 1993). Given that Pmar1 rescues the early specification of micromeres after removal of β-catenin from nuclei and Pmar1 controls expression of the two induction signals, we next asked if SoxB1 clearance occurs in the animal pole in response to *pmar1* expression by transplanted micromeres or mesomeres. Micromeres pretreated in several ways were transplanted to the animal poles of early fourth cleavage hosts. Control micromeres induced the secondary axis as expected. Micromeres expressing Δ-*cadherin* failed to induce a secondary axis when transplanted to the animal pole as shown earlier (Logan et al., 1999). If normal micromeres or Δ-*cadherin*-expressing micromeres also expressing *pmar1* are placed at the animal pole, the host cells nearby eliminate SoxB1 (Fig. 6). Similarly, mesomeres expressing *pmar1* have the ability to induce SoxB1 nuclear elimination if placed at the animal pole of control embryos (Fig. 6). We conclude that *pmar1* expression is upstream of a signal that induces ectopic clearance of SoxB1 from cells at the animal pole. It is likely that this signal and its response are at least a portion of secondary axis induction, a test for which we have a molecular assay. Most embryos under these conditions go on to make ectopic skeletons and a secondary gut, indicating that *pmar1* expression provides micromeres or mesomeres with the capability to induce a secondary axis.

Discussion

Use of blastomere transplantation to test gene regulatory network logic

The GRN for micromere specification and function published by Oliveri et al. (2002) was based entirely on whole embryo data. Several kinds of experimental information underlie the provisional GRN. The most basic is the time and place of expression of all the genes that participate in the network. Causal relationships within the GRN emerged from extensive QPCR observations on the specific consequences to other genes after perturbing regulatory genes specifically in micromeres and their descendants. WMISH experiments were carried out to reveal the effects on other genes of the spatial misexpression of *pmar1*. The *pmar1* gene encodes a transcriptional repressor normally active only in micromeres. Its function is proposed to inactivate an otherwise globally active repressor(s), thereby allowing micromere-specific genes to be expressed only in that lineage. To those data, the experiments in this manuscript add direct biological tests of predictions of the GRN. The correct placement of three major functions of *pmar1* in micromeres is supported in micromere transplantation experiments. Further, ectopic activation of *pmar1* transfates other cells of the embryo to carry out the micromere program showing that expression *pmar1* is sufficient for those cells to become PMCs.

Fig. 7 is a current version of the micromere specification GRN, modified somewhat from that shown in Oliveri et al. (2002), and simplified to focus on the results of tests in the present set of experiments. None of the other modifications, which are based on more recent data (http://www.its.caltech.edu/~mirsky/qper.htm), affect any aspects of the GRN directly relevant to the present work. The GRN is essentially a logic map, intended to display the regulatory

activators that account for the specific functions executed by the micromere lineage up to the point of ingression (20 h in *S. purpuratus*). There are three such functions: the expression of the "early signal" (ES) required for veg2 endomesoderm specification; the subsequent expression of the Delta signal required for specification of pigment and other mesoderm cell types from veg2 blastomeres lying adjacent to the micromeres; and the installation of a skeletogenic regulatory state in the micromeres.

The several specific features of the GRN shown in Fig. 7 that were directly challenged by the experiments in this manuscript are: (1) the prediction that expression of the *pmar1* gene is all that is required to transduce the essential β-catenin input into the micromere specification system; (2) the prediction that expression of the *pmar1* gene is all that is required to cause any cell in the embryo to execute all three of the functions normally carried out by the micromere lineage; (3) the corollary prediction that no prelocalized micromere "factors" or other features are needed downstream of *pmar1* gene for it to execute its role in micromere specification, nor are there any signals from the veg2 cells adjacent to the micromeres required; and (4) the prediction implicit in the GRN of Fig. 7 that, once *pmar1* is expressed, micromere specification carries on autonomously.

Tests of the predictions

The experiments of Fig. 1 show that expression of *pmar1* in micromeres alone suffices to rescue skeletogenic specification dependent on the β-catenin input. The experiment in Fig. 2 generalizes this same result to blastomeres normally fated to become ectoderm (mesomeres). Therefore, *pmar1* expression suffices to produce the skeletogenic regulatory state, and its downstream effector functions, in cells of any lineage, position, and (normally) fate. This means that there can be no requirement for any other maternal micromere-specific factors downstream of *pmar1* itself.

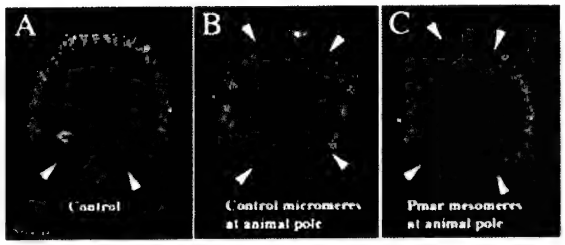
In the experiments of Figs. 1–3, pigment cells are formed in the host embryos from host embryo precursors. This induction depends on expression of *pmar1* (i.e., presence of *pmar1* mRNA) in transplanted micromeres also bearing *Δ-cadherin* (Figs. 1 and 3). No pigment cells are found if the transplanted micromeres express *Δ-cadherin* mRNA alone or if the micromeres are simply removed from an otherwise normal embryo. Mesomeres expressing *pmar1* also suffice to induce pigment cells if transplanted to a micromereless host. Therefore, as Fig. 7 predicts, *pmar1* expression suffices to permit inductively functional Delta expression, even in mesomeres. The dependence of *delta* expression in micromeres on β-catenin nuclearization is therefore due entirely to the fact that the β-catenin input is needed for *pmar1* expression (Oliveri et al., 2002).

There is extensive evidence that *delta* expression lies downstream of *pmar1*. It cannot lie upstream because the *delta* gene is expressed much later than is *pmar1*. Nor is the *delta* gene wired in parallel with *pmar1* with independent inputs because perturbation of *pmar1* expression specifically affects *delta* expression in the embryo and also at the *cis*-regulatory level (Oliveri et al., 2002; unpublished data from our laboratory). Nor can *delta* be upstream of the ES because this signal is passed at a stage prior to the time when the *delta* gene is activated (Ransick et al., 1995). A recent experiment of Sweet et al. (2002) shows that *delta* expression by mesomeres endows these cells with the ability to generate endoderm and skeleton, as well as mesoderm. However, blockade of *delta* translation with a Morpholino antisense oligo shows that, in a normal context, both skeleton formation and endoderm specification proceed normally (Sweet et al., 2002). The animal cap experiment thus indicates a train of regulative respecifications, and does not in fact conflict with the evidence underlying the present experiment, which places *pmar1* upstream of *delta,* and *delta* specifically responsible for the induction of pigment cells (Oliveri et al., 2002; Davidson et al., 2002; Sweet et al., 2002).

Figs. 4–6 concern the dependence of the ES on *pmar1* expression, also indicated in Fig. 7. Clearance of SoxB1 from endomesoderm nuclei is used as an early index of the effect of the ES on the veg2 endomesodermal precursors (unpublished data). Fig. 4 shows that this clearance fails in micromereless embryos and in embryos to which micromeres expressing Δ-cadherin mRNA are transplanted. But again, transplanted micromeres expressing both Δ-cadherin mRNA and *pmar1* mRNA lead to SoxB1 clearance in the adjacent cells (Fig. 4), and a normal archenteron is also induced to form. Very dramatically, SoxB1 clearance can even be induced ectopically by transplanting mesomeres expressing *pmar1* mRNA to the animal pole (Fig. 6). In Fig. 5, it is similarly demonstrated that *endo16,* an endomesodermal marker gene, is activated in mesomeres adjacent to clones of cells producing Pmar1 from a genetic expression construct. The ES mRNA is therefore also likely to be an early transcriptional product of the micromeres. Combined, these experiments show that expression of the ES in any cell requires *pmar1* expression; that the need for the β- catenin input for ES expression is again by way of the *pmar1* gene; and that no micromere-specific features downstream of *pmar1* expression are required for ES emission.

The micromere GRN

From these experiments, it can be concluded that no additional inputs downstream of *pmar1* gene expression are exclusive to micromeres. This means that the *cis*- regulatory element of *pmar1* is almost certainly the furthest upstream zygotic transcriptional apparatus in the micromere specification GRN. This gene is activated immediately upon the birth of the micromeres (Oliveri et al., 2002), so temporal considerations make it extremely unlikely that there is any additional micromere-specific zygotic transcriptional function upstream of it. The *pmar1 cis*-regulatory element is already in hand, and its inputs will soon be known. The



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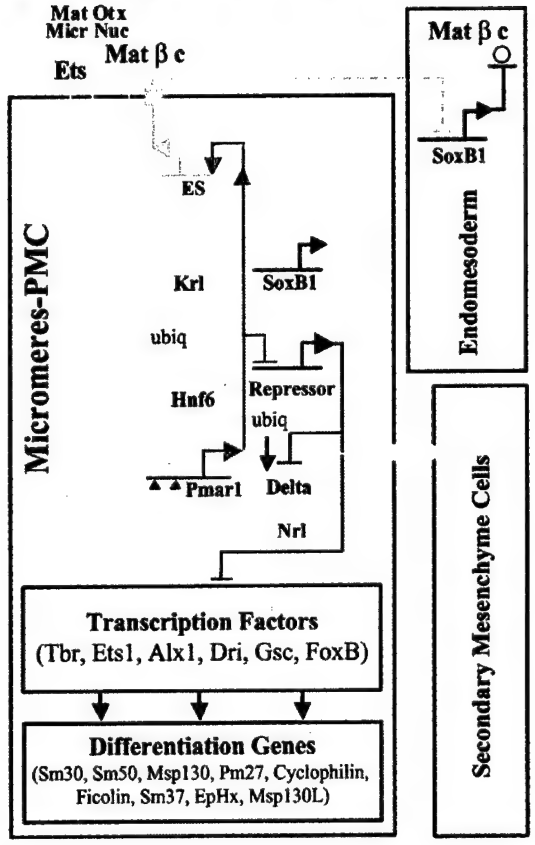

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Fig. 6. Pmar1-expressing micromeres or mesomeres have the capacity to induce a secondary axis if transplanted to the animal pole. (A) The vegetal pole of control embryos clears SoxB1 due to the presence of micromeres [now visible as PMCs (red) in the blastocoel (6/6 embryos examined)]. (B) If control micromeres are placed at the animal pole of a control embryo, they induce ectopic nuclear clearance of SoxB1 (6/6 embryos examined). (C) That same ectopic SoxB1 clearance is present at the animal pole if mesomeres expressing *pmar1* are transplanted to the animal pole (4/6 embryos examined). Green is immunostaining for SoxB1, and red is a specific PMC antibody. The hyaline layer outside the embryo is also stained nonspecifically with the red antibody (see Materials and methods for details).

Fig. 7. Micromere specification gene regulatory network. The network shown is modified from Oliveri et al. (2002). The diagram represents the "view from the genome": all the known interactions at gene level relevant for the micromeres-PMC specification. The colored lines indicate the interactions analyzed in this paper; the gray lines are not considered here. More details are available in the Web site: http://www.its.caltech.edu/~mirsky/endomes.htm. Arrows indicate activating positive functions and bars repressive interactions. The *pmar1* gene (red) is depicted in the middle of the GRN and is activated by maternal β-catenin (orange) and maternal Otx. Pmar1 is modeled to repress an unknown ubiquitous repressor(s) (blue). That repressor(s) prevents the micromere specification pathway from moving forward to activate specific early micromere-PMC transcription factors (*tbr, ets, alx1, dri,* and *foxB*). These, acting in different combinations, are responsible for the expression of the skeletogenic differentiation genes. The repressor also blocks the expression of *delta* (yellow), schematically represented as a membrane protein. The Delta ligand is responsible, through an interaction with Notch (yellow arrow), for the specification of the secondary mesenchyme cells (lower box on the right; Sherwood and McClay 1999; Sweet et al., 2002). When Pmar1 silences the repressor(s), *delta* is expressed and the SMC specification pathway is activated. Pmar1 also activates the production of the ES, illustrated as embedded in the schematic membrane (green). The double arrow from *pmar1* to the unknown gene coding for the ES indicates a possible indirect interaction. So far, Pmar1 has been shown to work as a repressor (Oliveri et al., 2002). The ES function is indirectly connected (green dashed line) to SoxB1 (black) clearance from the endomesoderm territory (upper right box). Thus, all three known early functions of the micromeres are enabled by Pmar1 expression.

β-catenin/TCF input is surely among them, as most likely is an Otx input (Davidson et al., 2002b; cf. QPCR data at http://www.its.caltech.edu/~mirsky/qpcr.htm). When completed, the specific inputs to which the *pmar1 cis*-regulatory element responds will define in molecular terms the maternal components localized in micromeres after the unequal 4th cleavage that are responsible for triggering activation of the micromere GRN.

Additional evidence that lies outside the scope of this paper explicitly places all the known skeletogenic subnetwork regulators, i.e., *tbr, ets, alx1, dri,* and *foxb,* downstream of the *pmar1* derepression system (QPCR perturbation experiments, *op cit.;* Amore et al., 2003; and much unpublished additional data of P.O. and E.H.D.). Thus, overexpression or knockout of these regulators affects skeletogenic functions (for *ets,* Kurokawa et al., 1999; for *dri,* Amore et al., op. Cit.; for *tbr,* Davidson et al., 2002a and Croce et al., 2001; plus other unpublished data). But these perturbations do not affect expression of the ES or the Delta signal, for example, as has been shown clearly in each case by morpholino knockouts followed by measurement of *delta* transcript level (data on QPCR web site, *op cit.*). As illustrated in Fig. 7, this is because the *delta* gene and the skeletogenic regulatory apparatus are hooked up in parallel to the *pmar1* derepression system.

A final point that emerges into the foreground of this work is that the GRN affords a direct, two-way transit between the evidential world of experimental embryology and the sequence-based logic relationships wired into the genomic regulatory system. This is of great interest for the design of future research. On the one hand, it breathes conceptual import into what has been a largely empirical branch of classical embryology. On the other hand, it provides a vivid means of testing the completeness of the logic predictions of the GRN, where the readout can be perceived directly, at the level of cell fate and function.

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LvTbx2/3: a T-box family transcription factor involved in formation of the oral/aboral axis of the sea urchin embryo

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SUMMARY

T-box family transcription factors have been identified in many organisms and are frequently associated with patterning events during embryonic development. With an interest in the molecular basis of patterning in the sea urchin embryo, we identified several members of the T-box family in *Lytechinus variegatus*. Here, we report the cloning and characterization of an ortholog of the Tbx2/3 subfamily, *LvTbx2/3*. To characterize the spatial distribution of LvTbx2/3 protein throughout sea urchin embryogenesis, a polyclonal antiserum was generated. Nuclear localization of LvTbx2/3 initiated at the mesenchyme blastula stage and protein was present into the pluteus stage. Localization was asymmetric throughout this period and costaining with marker genes indicated that asymmetry was about the oral/aboral (O/A) axis. Asymmetric distribution of LvTbx2/3 was observed in the aboral territories of all three germ layers. In the skeletogenic mesoderm lineage, LvTbx2/3 expression was dynamic because expression appeared initially in all skeletogenic mesenchyme cells (PMCs) but, subsequently, became refined solely to the aboral ones during skeletogenesis. To determine if the aboral expression of *LvTbx2/3* is linked between germ layers, and to place *LvTbx2/3* in the sequence of events that specifies the O/A axis, the effects of a series of perturbations to O/A polarity on *LvTbx2/3* expression in each germ layer were examined. Preventing the nuclear localization of β-catenin, pharmacological disruption of the O/A axis with $NiCl_2$, overexpression of *BMP2/4* and disruption of the extracellular matrix all blocked *LvTbx2/3* expression in all germ layers. This indicates that expression of *LvTbx2/3* in the aboral territories of each germ layer is a common aspect of O/A specification, downstream of the molecular events that specify the axis. Furthermore, blocking the nuclear localization of β-catenin, overexpression of *BMP2/4* and disruption of the extracellular matrix also prevented the oral (stomodael) expression of LvBrachyury (LvBrac) protein, indicating that the O/A axis is established by a complex series of events. Last, the function of LvTbx2/3 in the formation of the O/A axis was characterized by examining the phenotypic consequences of ectopic expression of *LvTbx2/3* mRNA on embryonic development and the expression of marker genes that identify specific germ layers and tissues. Ectopic expression of *LvTbx2/3* produced profound morphogenetic defects in derivatives of each germ layer with no apparent loss in specification events in those tissues. This indicates that LvTbx2/3 functions as a regulator of morphogenetic movements in the aboral compartments of the ectoderm, endoderm and mesoderm.

Key words: Sea urchin, T-box, Tbx2/3, Oral/aboral, Morphogenesis

INTRODUCTION

Cell-fate specification in the sea urchin embryo is achieved through an initial polarization of the unfertilized egg along the animal/vegetal (A/V) axis and, after fertilization, through cell-cell interactions within and between blastomere layers during cleavage and blastula stages. Such polarizations, signaling events and cell-cell interactions activate region-specific genes and segregate the 60-cell stage embryo into five embryonic territories: the small micromeres; the skeletogenic mesenchyme; the vegetal plate endomesoderm; the oral ectoderm; and the aboral ectoderm (Davidson, 1998). Although there is much data about the development of the primary, A/V axis of the embryo (reviewed in Angerer and Angerer, 2000), less is known about the specification of the secondary, O/A axis in the sea urchin. In some species of sea urchins, the O/A axis can be predicted by the plane of first cleavage, whereas in others, the future axis cannot be related to the position of cleavage planes until the third cleavage or later (Cameron et al., 1989; Henry et al., 1992). Signaling events seem also to be involved (Wikramanayake et al., 1995; Wikramanayake and Klein, 1997).

Several genes are expressed asymmetrically about the O/A axis in the endoderm, mesoderm and ectoderm, but no genes have yet been identified that reflect the oral/aboral polarity extending between all three. In the endoderm, asymmetrically

localized gene products include apical LvNotch protein, which is enriched on the aboral side (Sherwood and McClay, 1997). In the nonskeletogenic mesoderm, CyII actin is distributed orally, and OrCT, CAPK and Pl103 aborally (Miller et al., 1996; Rast et al., 2002). O/A patterning has been studied most in the ectoderm and, thus, many gene products with asymmetric distribution about the axis have been identified. Aboral genes include *Spec1* and *Spec2* (Lynn et al., 1983), *CyIIIa* actin (Cox et al., 1986), *arylsulfatase* (Sasaki et al., 1988), *Hox8/Hbox1* (Angerer et al., 1989), *SpMTA* (Nemer et al., 1995) and *P3A2* (Calzone et al., 1991); whereas oral genes include EctoV (Coffman and McClay, 1990) *Otx* (Li et al., 1997a; Yuh et al., 2002), *SpCOUP-TF* (Vlahou et al., 1996), *BMP2/4* (Angerer et al., 2000), *PlOtp* (Di Bernardo et al., 1999), *Brachyury* (Gross and McClay, 2001) and *goosecoid* (Angerer et al., 2001).

Based on the expression patterns and likely function of members of the family of T-box genes in regionalization of body plans in other organisms, we hypothesized that they might play a similar role in the sea urchin embryo. Members of the T-box family of transcription factors have been identified in all metazoan organisms in which they have been sought (reviewed by Papaioannou and Silver, 1998; Smith, 1999). T-box genes are characterized by homology to the DNA-binding domain of Brachyury, the founding member of the T-box gene family. The T-box encompasses ~180 amino acids and can be located anywhere in the protein (Kispert and Hermann, 1993). T-box proteins share little homology outside this region and it is in the T-box that the specificity for target promoters resides (Conlon et al., 2001). The T-box family includes transcriptional activators such as *Brachyury*, *Tbx5*, *VegT* and *Eomesodermin* (Kispert et al., 1995; Horb and Thomsen 1997; Horb and Thomsen, 1999; Ryan et al., 1996) as well as transcriptional repressors such as *Tbx2* and *Tbx3* (Carreira et al., 1998; He et al., 1999). The importance of T-box genes in development is underscored by their involvement in a variety of human pathologies, including that of *Tbx5* in Holt-Oram syndrome (Basson et al., 1997; Li et al., 1997b), *Tbx1* in DiGeorge syndrome (Jerome and Papaioannou, 2001; Merscher et al., 2001), *Tbx3* in ulnar-mammary syndrome (Bamshad et al., 1997) and, possibly, *Tbx2* in breast cancer (Jacobs et al., 2000).

Here, we report the identification and characterization of *LvTbx2/3*, a member of the *Tbx2/3* subfamily of T-box genes, during development of the sea urchin embryo. LvTbx2/3 protein is concurrently expressed in the aboral territories of the endoderm, mesoderm and ectoderm. A series of perturbations to the molecular components that are thought to be involved in specifying the O/A axis revealed that the aboral distribution of LvTbx2/3 appears to be a common aspect of O/A specification in each of these tissues. Specifically, LvTbx2/3 expression is dependent on either β-catenin or genes downstream of β-catenin, and is prevented by ventralization with $NiCl_2$, overexpression of *LvBMP2/4* and disruption of the extracellular matrix (ECM). Thus, LvTbx2/3 is expressed downstream of, or relatively late in, the sequence of events that serve to specify this axis. That LvTbx2/3 expression can not be separated between the different tissues after perturbation indicates that O/A axis specification is linked in all three germ layers of the sea urchin embryo at the level of *LvTbx2/3* and may occur in parallel to the distinct specification events that give rise to the ectoderm, endoderm and mesoderm of the embryo. Ectopic expression of *LvTbx2/3* supports this conclusion in that universal expression of *LvTbx2/3* profoundly affects the morphogenesis of ectoderm, endoderm and mesoderm without altering specification events of embryonic territories. Combined with the loss of expression of *LvTbx2/3* after perturbation of O/A specification, these results indicate that *LvTbx2/3* may be a downstream component of the O/A axis program that is involved specifically in morphogenesis of aboral territories in the embryo.

MATERIALS AND METHODS

Animals

Sea urchins (*L. variegatus*) were obtained from either Susan Decker (Hollywood, FL) or Jennifer Keller (Duke University Marine Laboratory). Gametes were harvested and cultured at 23°C as described (Hardin et al., 1992).

Cloning an *LvTbx2/3* fragment

Degenerate primers were designed that corresponded to the amino acids YIHPDSP (forward)/AVTAYQN (reverse) and used in a PCR reaction with cDNA template prepared from mid gastrula poly(A)$^+$ mRNA. PCR conditions were 45 cycles of 96°C for 60 seconds, 40°C for 60 seconds, 72°C for 2 minutes 45 seconds. The amplified, 234 bp products were gel purified, cloned into the pGEMT vector (Promega) and sequenced bidirectionally (Duke Sequencing Core). Clones were identified as PCR products of *LvTbx2/3* by BLAST search.

cDNA library screens

Screens were performed essentially as described (Gross and McClay, 2001) with hybridizations performed at 55°C in 0.5 M $NaHPO_4$ pH 7.2, 1 mM EDTA, 7% SDS, after Church and Gilbert (1984). After rescreens, nine clones were excised, sequenced and identified as *LvTbx2/3* fragments. A full-length open-reading frame was defined by overlapping individual fragments.

Northern analysis

Northern blotting (RNA gel blot hybridization) for *LvTbx2/3* was performed as described (Gross and McClay, 2001). Blots were given two 5 minute washes with 6× SSPE, 0.5% SDS at room temperature, one 45 minute wash with 1× SSPE, 0.1% SDS at 37°C, and one 45 minute wash with 1× SSPE, 0.1% SDS at 50°C. After washing, the blot was wrapped in plastic wrap and placed on film for 72 hours at –70°C with an intensifying screen. It was then stripped in 50% formamide, 6× SSPE for 30 minutes at 65°C and reprobed as above with an *L. pictus* ubiquitin fragment as a loading control.

Antibody production

LvTbx2/3 fusion protein was expressed following PCR amplification of a *Bam*HI-*Xho*I fragment of *LvTbx2/3* (encoding amino acids 11-339) and subcloning into the pGEX4T-1 glutathione S-transferase (GST) expression system (AmershamPharmacia Biotech). Expressed, affinity-purified protein (80 μg) was mixed 1:1 with Freund's complete adjuvant and injected into each of three guinea pigs (Charles River, Raleigh, NC). Animals were boosted with 80 μg protein mixed 1:1 with incomplete Freund's adjuvant after 21, 42 and 70 days. Bleeds were performed 31, 53 and 80 days after the last injection and serum isolated as described (Harlow and Lane, 1988).

Western analysis

1500 late-gastrula embryos were homogenized in the presence of protease inhibitors, boiled and run on a 10% SDS-PAGE gel. Protein was blotted onto nitrocellulose, blocked overnight at 4°C

in 2% milk, 1% bovine serum albumin (BSA) TBST and probed for 1.5 hours at room temperature with a 1:1000 dilution of either α-Tbx2/3 or preimmune serum in 2% milk, 1% BSA TBST. The blot was washed three times with TBS before applying goat α-guinea pig HRP-tagged secondary antibody (Jackson Immunoresearch Laboratories) at 1:5000 for 1 hour at room temperature. Labeled proteins were visualized by ECL (AmershamPharmacia Biotech).

Immunolocalization and image analysis

Embryos were fixed in 2% paraformaldehyde, 60% artificial sea water (ASW) for 10-12 minutes at room temperature, before being permeablized for 60 seconds with ice cold, 100% methanol. They were then washed three times with PBS, blocked 10-20 minutes in PBS, 4% normal goat serum (NGS; GibcoBRL) and incubated overnight at 4°C in primary antibody, 4% NGS. After washing four times in PBS, they were blocked as above and incubated for 60 minutes at room temperature in secondary antibody, 4% NGS (either Cy3 or Cy5-conjugated; Jackson Immunoresearch Laboratories). Embryos were then washed four times in PBS and mounted in 70% glycerol. LvTbx2/3 and LvBrac sera were diluted 1:500 for all images. Undiluted supernatants of monoclonal antibodies (mABs) 5a7 (EctoV), 5c7 (Endo1) and 295 were used with the above fixation and incubation conditions. All images were obtained using a 40× Plan-Neofluar oil-immersion objective (NA=1.3) on a Zeiss laser-scanning confocal microscope (Carl Zeiss, Thornwood, NY) mounted on a Zeiss Axiovert inverted microscope. Where necessary, 1 μm sections from single label images were rendered into 3D projections using Zeiss confocal software. Double labeled images were taken sequentially using appropriate filters and subsequently overlayed using Adobe Photoshop 5.0.

Chemical treatments

Treatment of embryos with either $NiCl_2$ or β-aminopropionitrile (βAPN) were performed as described (Hardin et al., 1992; Wessel and McClay, 1987).

Generation of LvTbx2/3 constructs

Full-length *LvTbx2/3* was generated by subcloning fragments from individual excised cDNA clones obtained in library screening (details available on request). For ectopic overexpression studies, an *SpOtx* 5′ UTR plus the first five amino acids of *SpOtx* was cloned in frame, 5′ to the *LvTbx2/3* translation-start site. This leader sequence has been demonstrated to provide an excellent translation start site for mRNA constructs in the sea urchin (Sherwood and McClay, 1999). All clones were sequenced bidirectionally to verify fidelity.

mRNA preparation and injection

ΔLvG-cadherin and *LvBMP2/4* were linearized and injected as described (Logan et al., 1999; Angerer et al., 2000). *LvTbx2/3* was linearized with *XhoI* and used as a template to generate in vitro-transcribed 5′ capped mRNA using the T3 mMessage mMachine kit (Ambion). Concentrations of mRNA were determined by spectrophotometry, and by comparison to known amounts of RNA using both gel electrophoresis and dotting onto a 0.6% agarose gel.

Quantitative PCR (QPCR)

RNA was isolated using Trizol (Invitrogen). Reverse transcription reactions were performed using oligo dT priming and MMLV-reverse transcriptase (Gibco). Reactions were purified using a PCR-purification kit (Qiagen). QPCRs were performed using Roche LightCycler Fast Start Master SYBR as manufacturers instructions. Primers used were ubiquitin (Rast et al., 2000) and LvTbx2/3. A Tbx2/3 plasmid was used to generate a standard curve for quantification, and ubiquitin was used to normalize the cDNA samples. Each time point was determined from two independent batches, and each reaction was confirmed by gel electrophoresis.

RESULTS

Identification of a *Tbx2/3* subfamily member in the sea urchin

LvTbx2/3 was PCR amplified from a mid-gastrula stage cDNA pool using degenerate oligonucleotides that correspond to evolutionarily conserved regions of the DNA-binding domain of other T-box proteins. Cloning and sequencing of the amplified fragment identified it as a *L. variegatus Tbx2/3* ortholog (*LvTbx2/3*). A mid-gastrula cDNA library was then screened and nine *LvTbx2/3* cDNA clones recovered. Alignment of the sequences of these clones defined the full coding region of the gene. *LvTbx2/3* encodes a 637 amino acid protein, based on the predicted open reading frame from the primary sequence data (Fig. 1A; GenBank accession number AY120889). Supporting *LvTbx2/3* as a member of this T-box subfamily, LvTbx2/3 aligns in a phylogenetic tree of Tbx2/3 subfamily proteins (Fig. 1B).

Northern-blot analysis of *LvTbx2/3* mRNA revealed that a 5.37 kb message appears first at the mesenchyme blastula stage and that it is present throughout the pluteus stage (Fig. 2). The highest concentrations of mRNA were observed during gastrula stages. Quantitative PCR analysis of the *LvTbx2/3* RNA corresponds well with the Northern-blot data. No *LvTbx2/3* mRNA is present in the egg but low concentrations start to be detected at the hatched blastula stage. In the mesenchyme blastula there are ~200 copies of *LvTbx2/3* mRNA per aboral cell and this level is retained until the early prism stage when the number of copies per aboral cell drops. To characterize the temporal and spatial distribution of LvTbx2/3 protein, a polyclonal antiserum was generated in guinea pigs against recombinant LvTbx2/3 (amino acids 11-339 fused to GST). This serum was tested for immunoreactivity by protein analysis on SDS-PAGE gels and whole-mount immunofluorescent staining of fixed embryos. Western blots of protein extracts from late gastrula were probed with LvTbx2/3 polyclonal and preimmune sera to ascertain specificity (Fig. 3). Two immunoreactive bands were observed when blots were probed with LvTbx2/3 serum, one of ~70 kDa and one of 35 kDa. The 70 kDa band corresponds with the predicted size of LvTbx2/3 from primary sequence data (637 amino acids). The 35 kDa band was also recognized when blots were probed with preimmune serum, indicating that it is a nonspecific antigen. Whole-mount immunofluorescent analysis of fixed embryos stained with preimmune serum did not result in any distinct staining pattern at any stage examined (Fig. 4C). Additionally, embryos stained with LvTbx2/3 serum after preincubation with recombinant LvTbx2/3 protein did not stain positively at any stage examined (Fig. 4D).

Throughout development, the spatial distribution of LvTbx2/3 is asymmetric about the O/A axis in the endoderm, mesoderm and ectoderm

Fig. 4A,B shows two different orientations of prism-stage embryos costained with anti-LvTbx2/3 antiserum (red) and 5a7 mAB (green). 5a7 recognizes EctoV, a protein that is localized to the foregut and oral ectoderm (Coffman and McClay, 1990). LvTbx2/3 was localized to the nucleus, as expected given its role as a transcription factor. A striking asymmetry of LvTbx2/3 distribution was observed in the ectoderm, endoderm and skeletogenic mesoderm at this stage of

development. EctoV and LvTbx2/3 were present in complementary patterns, indicating that this asymmetry is about the O/A axis of the ectoderm and that LvTbx2/3 is restricted solely to the aboral territories of the embryo. The aboral distribution of LvTbx2/3 in the endoderm and mesoderm is apparent in Fig. 4B where the protein is clearly localized to the aboral regions of the archenteron and skeletogenic mesoderm. As such, LvTbx2/3 is the first marker of O/A polarity expressed in the derivatives of all three germ layers of the sea urchin embryo. Additionally, the LvTbx2/3 characterization reported here is, to our knowledge, the first report of protein expression for a non-Brachyury T-box gene in any organism.

The ectoderm, endoderm and mesoderm are all specified prior to LvTbx2/3 expression. Because LvTbx2/3 was distributed in a subset of cells in each of these tissues, we next characterized the temporal details of LvTbx2/3 protein expression (Fig. 5). LvTbx2/3 was localized to the nucleus at all stages examined. At mesenchyme blastula stage, LvTbx2/3 protein was observed in cells of the presumptive endoderm and ectoderm but not the mesoderm, as neither the ingressed skeletogenic nor the presumptive nonskeletogenic mesoderm expressed LvTbx2/3 protein when these territories were defined by marker genes (data not shown). A view of the vegetal surface of an early-gastrula stage embryo is shown in Fig. 5D. LvTbx2/3 was present at high concentrations in the presumptive endoderm and the ectoderm that surrounds the blastopore, whereas invaginated tissues contained much less protein. Asymmetric distribution in the endoderm and ectoderm continued through mid-gastrula stage (Fig. 5E,F). Between mid-gastrula and late-gastrula stages, LvTbx2/3 started to be expressed in cells of the skeletogenic mesenchyme lineage and the asymmetric localization in the invaginated endoderm became more apparent (Figs 5, 6). LvTbx2/3 protein in early and late-plutei embryos is shown in Fig. 5I-K. From an animal view of an early pluteus embryo that has been optically sectioned and

A

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1    ATG AAA CCG GCA TCG AAC GAC CAC CAC ACG ATG GCC TAT GCA CCT ATA CTG CCG CCT CGT CTC
       M   K   P   A   S   N   D   H   H   T   M   A   Y   A   P   I   L   P   P   R   L
64   AGC GAC TTC TCC GTC AAC TCC CTC CTC ACA CCG CCC CAA TTC TTC CCG GGG ATG TTC CGC GGA
       S   D   F   S   V   N   S   L   L   T   P   P   Q   F   F   P   G   M   F   R   G
127  CAG GCC TGT TTG CCC GGC GCA GGG CTG CCG GGC TTT CCC CTG CCG AAA TTC GGT GAA CAT CCA
       Q   A   C   L   P   G   A   G   L   P   G   F   P   L   P   K   F   G   E   H   P
190  GCG GGG TAC TCC CCG CAT GAC TTG TTG GCA GCA CAC GCT CAT CGC TCA GCA TTG GGC CCC TTA
       A   G   Y   S   P   H   D   L   L   A   A   H   A   H   R   S   A   L   G   P   L
253  CAC CCC ATG GAA ACA CAG AGC GAC GAT TCG GAT GAT CCA CAA GTT ACA CTT GAA TCT AAA GAA
       H   P   M   E   T   Q   S   D   D   S   D   D   P   Q   V   T   L   E   S   K   E
316  CTT TGG GAG AAA TTT CAC AAA AGA GGA ACG GAA ATG GTC ATC ACA AAA TCA GGC CGG CGG ATG
       L   W   E   K   F   H   K   R   G   T   E   M   V   I   T   K   S   G   R   R   M
379  TTC CCT TCT TTC AAA GTC CGT GTA TCT GGG CTG GAC AAG AAG GCC AAA TAC ATC CTT TTA ATG
       F   P   S   F   K   V   R   V   S   G   L   D   K   K   A   K   Y   I   L   L   M
442  GAC ATC GTC GCC GCC GAC GAC TGC CGG TAC AAG TTT CAC AAT TCC CGC TGG ATG GTC GCT GGC
       D   I   V   A   A   D   D   C   R   Y   K   F   H   N   S   R   W   M   V   A   G
505  AAG GCC GAT CCC GAG ATG CCC AAA CGG ATG TAT ATA CAC CCG GAT TCT CCG AGC ACA GGG GAA
       K   A   D   P   E   M   P   K   R   M   Y   I   H   P   D   S   P   S   T   G   E
568  CAA TGG ATG CAG AAA TGT GTT TCA TTC CAT AAG CTC AAA CTC ACC AAT AAC ATC TCC GAC AAG
       Q   W   M   Q   K   C   V   S   F   H   K   L   K   L   T   N   N   I   S   D   K
631  CAT GGA TTC CAG ACC ATT CTG AAT TCG ATG CAC AAG TAC CAA CCT CGT TTC CAC ATT GTC AAG
       H   G   F   Q   T   I   L   N   S   M   H   K   Y   Q   P   R   F   H   I   V   K
694  GCC AAT GAC ATC CTC AAG CTT CCC TGG AGT CAA TTC AGG ACC TTC GTA TTC GTC GAG ACC GTC
       A   N   D   I   L   K   L   P   W   S   Q   F   R   T   F   V   F   V   E   T   V
757  TTC ATC GCT GTC ACT GCC TAT CAA AAC GAA AAG ATT ACG CAA CTT AAA ATA GAC TAC AAC CCA
       F   I   A   V   T   A   Y   Q   N   E   K   I   T   Q   L   K   I   D   Y   N   P
820  TTC GCT AAA GGT TTC AGA GAT ACT GGC GCA GGG AAA AGG GAA AAG AGG AAA TAC ATT GGT GCA
       F   A   K   G   F   R   D   T   G   A   G   K   R   E   K   R   K   Y   I   G   A
883  ACT GGT ACC TAT GAA ATC GAC CAT CGA GAC GGC GAT GAC ATC CCA AGC GAC CAG GAG GCC GAG
       T   G   T   Y   E   I   D   H   R   D   G   D   D   I   P   S   D   Q   E   A   E
946  GCC GCC GAG GTC AGC ACA ACC AGC AAC GAC AGG CAT GAC GAA AGA GGT CAT TCG TCA CAC GAG
       A   A   E   V   S   T   T   S   N   D   R   H   D   E   R   G   H   S   S   H   E
1009 CTT GCA AGG CTA GCC AGC GAG GGC CGC CTG AAC GGA CCT GGC CTG AAC AAG TGT AAA CCC TCG
       L   A   R   L   A   S   E   G   R   L   N   G   P   G   L   N   K   C   K   P   S
1072 GAC ATG AAG GAA GGG CCA CAT GGA AGT TCA AGC TCC AAA GAT GAC GTA GAG ATG AGG GAT GTG
       D   M   K   E   G   P   H   G   S   S   S   S   K   D   D   V   E   M   R   D   V
1135 AGC TGT AAA GAC CAC GAG AGG AGG ATG GAG GGT AAA CAT AGA TTA AGT CAG GAT GAC AGT TCA
       S   C   K   D   H   E   R   R   M   E   G   K   H   R   L   S   Q   D   D   S   S
1198 ATT GAC AAG AAA ACC GAT CAC AAT GAG CGA TCA GAT TCG CGG AAA AGT GAC GGG CCC AGT TCA
       I   D   K   K   T   D   H   N   E   R   S   D   S   R   K   S   D   G   P   S   S
1261 AGA CTT TCT CCT CCA AGT CTA CAC CTT GGT TCT GCC GGC TCA TCC TTC TCG TCA TTG CAC GGT
       R   L   S   P   P   S   L   H   L   G   S   A   G   S   S   F   S   S   L   H   G
1324 TCC CAT CCA CCT GTT GTG ACG CCA ATC TAC CCC ACA CCT CAG CAG TTA TTT CTC AAT CCC CAT
       S   H   P   P   V   V   T   P   I   Y   P   T   P   Q   Q   L   F   L   N   P   H
1387 GCG CTA CAT GGT GCT GTA CCA GGA CTT GGA GCA ATG CAT CAC ATG TTA CCC CTC CCA AGC AGC
       A   L   H   G   A   V   P   G   L   G   A   M   H   H   M   L   P   L   P   S   S
1450 TCC TCC CAT TCC CCT TCA GGA CAT CCT AGT TAT TTA GAC GCC CAT CCT TTC GCG TTT GGA GCA
       S   S   H   S   P   S   G   H   P   S   Y   L   D   A   H   P   F   A   F   G   A
1513 GCT CAT GCT TCA GGA CTC CTT TCC TCG CAA GGT GGC GCC GCC AGC TTT GGC AGC CTC TAT TCA
       A   H   A   S   G   L   L   S   S   Q   G   G   A   A   S   F   G   S   L   Y   S
1576 GAG GCC GCC GCC CTT AGC TCA ATG TAT GCC AGC AAC CCG TGT ACT AGT GCA ATA TTA AAT GGA
       E   A   A   A   L   S   S   M   Y   A   S   N   P   C   T   S   A   I   L   N   G
1639 CAT CCA AGA TTA AGG TTC TCA CCT TAT CAC CTA CCA GTC ACC AGC ACG ACT ATG GTC ACC ACT
       H   P   R   L   R   F   S   P   Y   H   L   P   V   T   S   T   T   M   V   T   T
1702 GCT AAC CCT CTA GCC ACA CCT ATC CCA TAC GAA AGC GCG TTA CAT TCA TCA CTC TCA GCG TTT
       A   N   P   L   A   T   P   I   P   Y   E   S   A   L   H   S   S   L   S   A   F
1765 GGT GGG TCA TCA CTG CTC ACG CCG GCC TCG GCA TCC ACT TCA CCG ACA TCG TCG TCA TCT TCA
       G   G   S   S   L   L   T   P   A   S   A   S   T   S   P   T   S   S   S   S   S
1828 CTT CCA GCC AGT AAA GAC GTG CCG ACG TCG CCT GCG AGG TCG GTC AGC GCT GCC ACA AAC GAA
       L   P   A   S   K   D   V   P   T   S   P   A   R   S   V   S   A   A   T   N   E
1891 CTT CAA AGC ATA CAA AAG ATG GTC AGT GGA CTC GAT AAA ACA CAA AAA TGA
       L   Q   S   I   Q   K   M   V   S   G   L   D   K   T   Q   K   .
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B

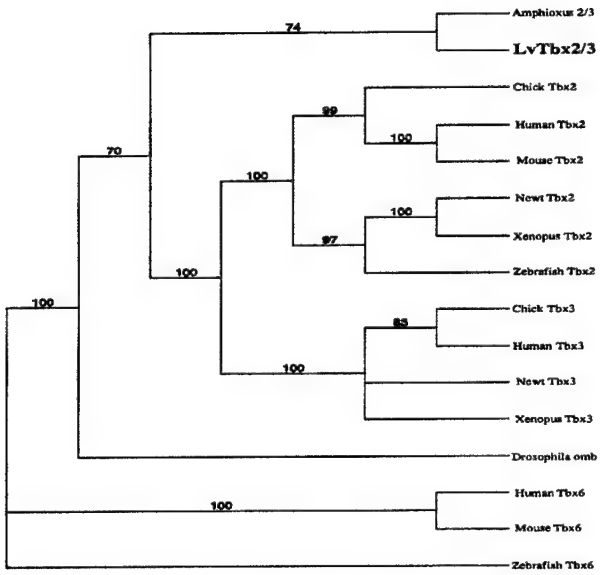

Fig. 1. (A) Nucleotide and predicted amino acid sequences of *LvTbx2/3*. (B) Phylogenetic tree of LvTbx2/3, Tbx2, Tbx3 and Tbx2/3 orthologs from other organisms generated by the neighbour-joining method. Bootstrap values indicated on nodes.

projected so that the animal-most ectoderm is removed, asymmetric distribution of LvTbx2/3 was observed in the ectoderm of the embryo and in the archenteron (Fig. 5I). A vegetal projection of a similarly staged pluteus embryo revealed that LvTbx2/3 is present in the ectoderm that surrounds the anus and was very strong in the distal-most portions of the extending embryonic arms (Fig. 5J). Although the concentration of LvTbx2/3 began to decline at the late pluteus stage, it was still observed asymmetrically in the ectoderm, endoderm and skeletogenic mesenchyme (Fig. 5K).

When LvTbx2/3 first appeared in the skeletogenic mesoderm, it was present in all of the PMCs (Fig. 5G,H and data not shown). However, as development proceeded to the prism and pluteus stages, LvTbx2/3 became restricted to the aboral PMCs (Figs 4, 5). The spatial and temporal aspects of LvTbx2/3 expression in the skeletogenic mesenchyme were further characterized to determine precisely when this restriction occurs (Fig. 6). Fig. 6A shows the initial, panskeletogenic mesoderm distribution of LvTbx2/3. Two different levels of confocal projections from the same prism-stage embryo are shown (Fig. 6B,C). The asymmetric distribution of LvTbx2/3 protein is clearly confined to the aboral PMCs and not present in the ventrolateral clusters. An oblique view of another late-prism stage embryo stained for LvTbx2/3 (red) and 5a7 (green) reinforced this observation, because LvTbx2/3 persists in the aboral ectoderm and endoderm of the embryo whereas no expression is observed in the ventrolateral clusters of PMCs (Fig. 6D). In early-pluteus stage embryos, asymmetric distribution of LvTbx2/3 persists in the PMCs, endoderm and ectoderm (Fig. 6E,F). Thus, in the skeletogenic mesoderm LvTbx2/3 is restricted to the aboral PMCs between late gastrula and early prism stages at the time when skeletal patterning begins to shape the spicule skeleton.

Fig. 2. Developmental northern blot of *LvTbx2/3* expression. Poly(A)$^+$ RNA (3 μg per lane) was loaded (calculated by OD_{260}). Loading was verified by probing the blot with a ubiquitin fragment from *L. pictus* (data not shown). Egg; 60 cell stage; MB, mesenchyme blastula; EG, early gastrula; LG, late gastrula; PR, prism; PL, pluteus larva.

LvTbx2/3 in the sequence of O/A axis specification and patterning

The striking asymmetry of the distribution of LvTbx2/3 about the O/A axis in the endoderm, ectoderm and mesoderm raises the possibility that O/A polarity might be either established or maintained by the same molecular component(s) in all three germ layers of the embryo. To place LvTbx2/3 in the framework of specification pathways and patterning events that impinge on the formation of the O/A axis, and to gain further insights into the mechanisms of O/A axis specification, the distribution of LvTbx2/3, 5a7 (Fig. 7A) and LvBrac (Fig. 7B) were examined under a variety of perturbations to this axis.

β-catenin/vegetal signaling in O/A patterning

The influence of β-catenin and β-catenin-dependent signaling on LvTbx2/3 expression was assayed first. Injection of a construct that encodes the cytoplasmic tail of the sea urchin ortholog of E-cadherin, *LvG-cadherin* (*ΔLvG-cadherin*) (Logan et al., 1999), serves as a 'sink' for cytoplasmic β-catenin by binding to it and preventing nuclear translocation and gene activation. Such embryos develop without endoderm

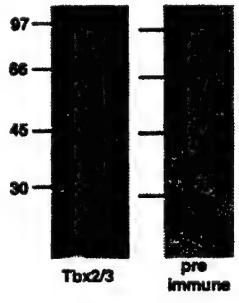

Fig. 3. Tbx2/3 polyclonal and preimmune sera controls. Western-blot analysis of protein extracts from late gastrula (1500 embryos) using polyclonal LvTbx2/3 and preimmune serum. In blots probed with LvTbx2/3, two immunoreactive bands appear, one of ~70 kDa and one of 35 kDa. A 35 kDa band was also recognized by preimmune serum, indicating the presence of a nonspecific antigen.

or mesoderm, and β-catenin depletion eliminates the O/A axis in the ectoderm (Wikramanayake et al., 1998; Logan et al., 1999). Expression of this construct resulted in uniform expression of EctoV (Fig. 7C) and prevented the expression of LvTbx2/3 (Fig. 7D). LvBrac and *SpGsc* (*Goosecoid*), two gene products that normally localize to the stomodaeum, are also not expressed when the nuclear translocation of β-catenin is prevented (Gross and McClay, 2001; Angerer et al., 2001). This indicates that the blockage of β-catenin nuclear localization prevents both aboral and oral (stomodael) specification.

Pharmacological perturbation of O/A patterning

Treatment of embryos with $NiCl_2$ at any point between the hatched blastula and early gastrula stages disrupts O/A patterning events (Hardin et al., 1992). Embryos perturbed in this manner are oralized, displaying defects in ectodermal patterning manifested by the formation of a circumferential stomodaeum around the animal pole, rather than at a localized

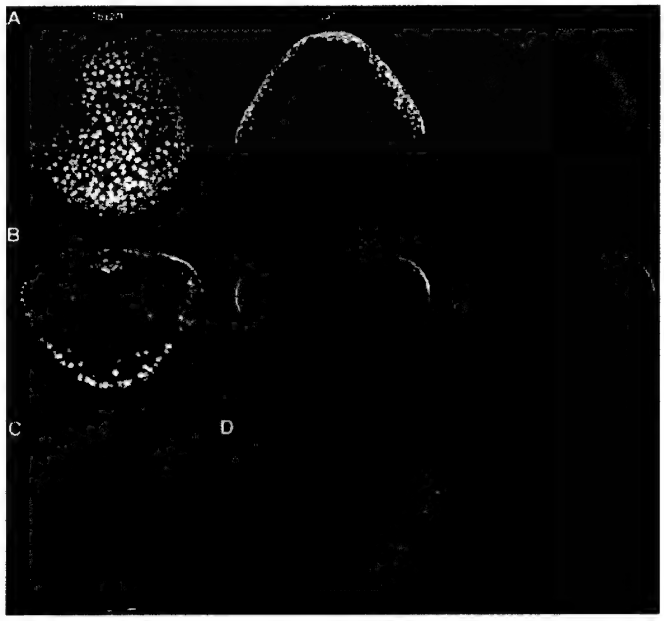

Fig. 4. Aboral distribution of LvTbx2/3 protein (red) in prism-stage embryos, demonstrated by co-staining with 5a7 (EctoV; green). The EctoV antigen is expressed from late gastrula stages onward, solely in the oral ectoderm and foregut. Prism-stage embryos viewed aborally (A) and in a vegetal cross-section (B). Complementary expression is observed, indicating that LvTbx2/3 is distributed asymmetrically about the oral/aboral axis, and localized in aboral territories of the endoderm, ectoderm and mesoderm. In many prism and pluteus-stage embryos, a patch of cilia on the oral surface crossreacts with Cy3 secondary antibodies nonspecifically, as observed in the LvTbx2/3 (red) images. (C) Whole-mount, immunofluorescent analysis of fixed embryos probed with preimmune serum. No nuclear staining is observed at any stage (early prism stage shown). (D) Whole-mount, immunofluorescent analysis of fixed embryos probed with with polyclonal LvTbx2/3 serum that had been preincubated with recombinant fusion protein. No nuclear staining is observed at any stage examined (early pluteus stage shown).

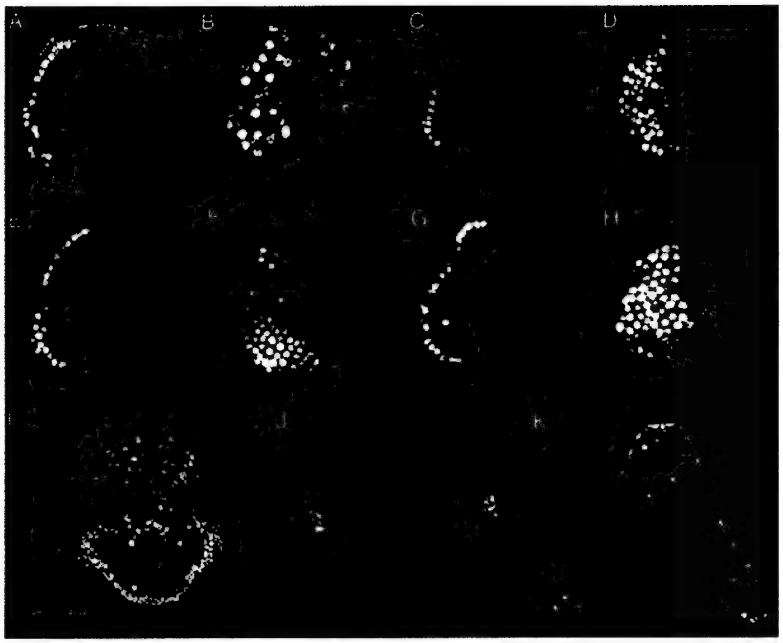

Fig. 5. LvTbx2/3 protein is asymmetric throughout embryonic development. Cross-section images (A,C,E,G,I,K) and surface projections (B,D,F,H,J). LvTbx2/3 expression first appears at the mesenchyme blastula stage and is distributed asymmetrically in the presumptive endoderm and ectoderm, as viewed in cross-section (A) and in a vegetal-surface view (B). Early-gastrula stage embryos in cross-section (C) and a vegetal view (D) maintain asymmetric distribution of LvTbx2/3 in the presumptive endoderm and ectoderm, whereas the endoderm and mesoderm that have invaginated into the blastocoel do not express protein. (E,F) Mid-gastrula stage embryo (cross-section and surface projection of the same embryo). LvTbx2/3 expression is maintained asymmetrically in the presumptive endoderm and ectoderm and is not present in invaginated endoderm or mesoderm. (G,H) Late gastrula distribution of LvTbx2/3 protein (cross-section and surface projection of the same embryo). Asymmetric expression is observed in the invaginated endoderm, the ectoderm and in all of the skeletogenic mesenchyme cells at this stage (also see Fig. 6). (I) Animal view of early pluteus embryo optically sectioned to remove the most superficial layers of ectoderm and expose the archenteron and stomodaeum. LvTbx2/3 is distributed asymmetrically in the surface ectoderm and the length of the archenteron. (J) Vegetal surface view. Distribution of LvTbx2/3 protein is asymmetric in the aboral ectoderm nuclei. High concentrations of LvTbx2/3 are also observed in the distal most nuclei of the extending pluteus arms. (K) Vegetal cross-section of a late pluteus embryo. Asymmetric distribution is maintained in cells of the ectoderm, endoderm and skeletogenic mesoderm.

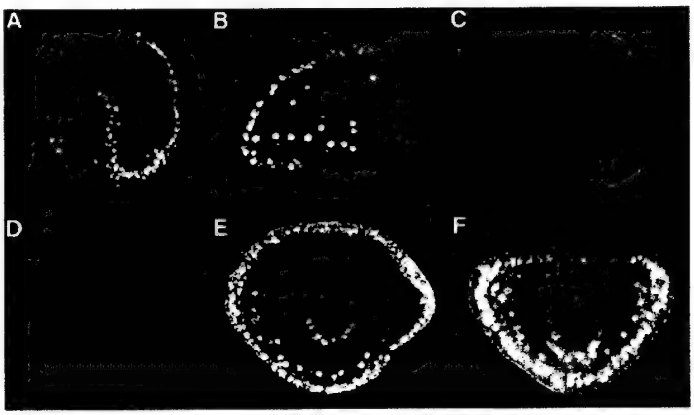

Fig. 6. Dynamic expression of LvTbx2/3 in the skeletogenic mesenchyme cells (PMCs). (A) Expression in PMCs begins at mid-late gastrula stage in all PMCs. (B,C) Two views of the same early-prism stage embryo. (B) Superficial view of the PMCs under the ectoderm. At this stage, asymmetric distribution of LvTbx2/3 is observed in the PMC lineage. (C) Deeper, cross-sectional view of the embryo in B co-stained for LvTbx2/3 (red) and EctoV (green). LvTbx2/3 localization in PMCs is in the aboral territory of the embryo. Note the clear, asymmetric distribution in the endoderm. (D) An oblique view of a late prism-stage embryo stained for LvTbx2/3 (red) and 5a7 (green). Distribution of LvTbx2/3 protein is limited to the aboral PMCs and is not present in the ventrolateral clusters that have begun to form triradiate spicules. Animal (E) and vegetal (F) cross-sectional views of early-pluteus stage embryos. LvTbx2/3 protein persists in the aboral PMCs and is not observed in the oral PMCs.

site, and the formation of ectopic spicule clusters. These animals express EctoV and LvBrac around their entire circumference except the vegetal plate (Hardin et al., 1992; Gross and McClay, 2001). Treatment of embryos with 1 mM $NiCl_2$ resulted in expression of LvBrac throughout the entire ectoderm (Fig. 7E) and elimination of LvTbx2/3 expression in all tissues (Fig. 7F).

BMP2/4 in O/A specification

Recent evidence indicates that an animally derived BMP2/4 ortholog affects O/A specification (Angerer et al., 2000). In situ analysis localizes *BMP2/4* mRNA to presumptive oral ectoderm at the hatching blastula stage. Ubiquitous overexpression of *BMP2/4* mRNA animalizes the embryo, causing it to form a ball of squamous epithelium whereas lower concentrations radialize the ectoderm of the embryo, as indicated by the formation of multiple clusters of spicules. At concentrations of *BMP2/4* mRNA that radialize the spicules, oral expression of LvBrac was prevented but vegetal LvBrac expression was normal (Fig. 7G). Aboral expression of LvTbx2/3 was not observed in the ectoderm, endoderm or skeletogenic mesoderm under such conditions (Fig. 7H). This indicates that ectopic expression of *BMP2/4* prevented the normal expression of *LvTbx2/3* in all three germ layers, and that O/A polarity in the ectoderm, mesoderm and endoderm is linked by some common genetic or molecular mechanism that is likely to be sensitive to changes in *BMP2/4* levels. The failure to observe stomodael LvBrac protein after ectopic expression of *BMP2/4* indicates that *BMP2/4* signals prevent the expression of a subset of genes in the oral ectoderm and do not uniformly oralize the embryo. Expression of LvBrac is normal in the vegetal blastopore region, indicating that, unlike in the stomodaeum, *LvBrac* regulation in this region is refractory to ectopic *BMP2/4* injected at this level.

The ECM in O/A patterning

Disruption of the ECM with βAPN, a drug that prevents collagen crosslinking implicates the ECM in O/A specification or maintenance. Embryos treated with βAPN do not gastrulate and do not express the aboral-ectoderm-specific *Spec1* gene (Wessel et al., 1989). The effects of ECM disruption on LvBrac and LvTbx2/3 expression was assayed (Fig. 7I,J). Neither stomodael LvBrac (Fig. 7I) nor aboral LvTbx2/3 (Fig. 7J) were expressed following treatment with βAPN. This indicates that an intact ECM is necessary for specification and/or maintenance of gene expression in both the oral and aboral territories of the ectoderm, not solely in the aboral territory as previously thought. Normal expression of LvBrac in the vegetal blastopore region indicates that this perturbation did not affect LvBrac regulation in this region.

Functional characterization of LvTbx2/3

The results of the perturbation studies detailed above place aboral *LvTbx2/3* expression downstream of several signals and specification events that are known to be involved in the formation of the O/A axis. To determine the role of *LvTbx2/3* in the formation of this axis, ectopic mRNA expression studies were performed. Ectopic *LvTbx2/3* expression produced drastic morphological defects in derivatives of all germ layers, suggesting that LvTbx2/3 functions in each germ layer (Fig. 8). Between 60-75% of embryos that ubiquitously express *LvTbx2/3* mRNA displayed severe morphological abnormalities 24-48 hours post-fertilization (three- to fivefold overexpression obtained following injection of 0.75-1 pg/pl of mRNA amounting to 600-1000 copies of *LvTbx2/3* mRNA per cell).

By 24 hours, control embryos injected with glycerol had formed a tripartite gut, characteristic skeletal structures and the embryonic shape appropriate for these stages of development (Fig. 8A,B). Embryos that expressed *LvTbx2/3* ectopically were often delayed in gastrulation but did invaginate endoderm and gastrulate normally several hours after controls (data not shown). At 24 hours post-injection, embryos injected with *LvTbx2/3* mRNA lacked normal skeletal rods and had a grossly mispatterned skeleton with several spicule clusters forming around the circumference of the embryo (Fig. 8C,D). Consistent with the observation that *LvTbx2/3* is downstream of both germ-layer specification and O/A patterning events, embryos that ectopically expressed *LvTbx2/3* had endoderm, ectoderm, skeletogenic mesoderm, pigment and blastocoelar cells. This indicates that germ-layer specification was not perturbed noticeably, rather, it is likely that aspects of patterning and later morphogenesis were affected. Because ectopic expression of T-box-family members might affect the function of other T-box proteins, these results must be considered cautiously. However, the interpretation that the

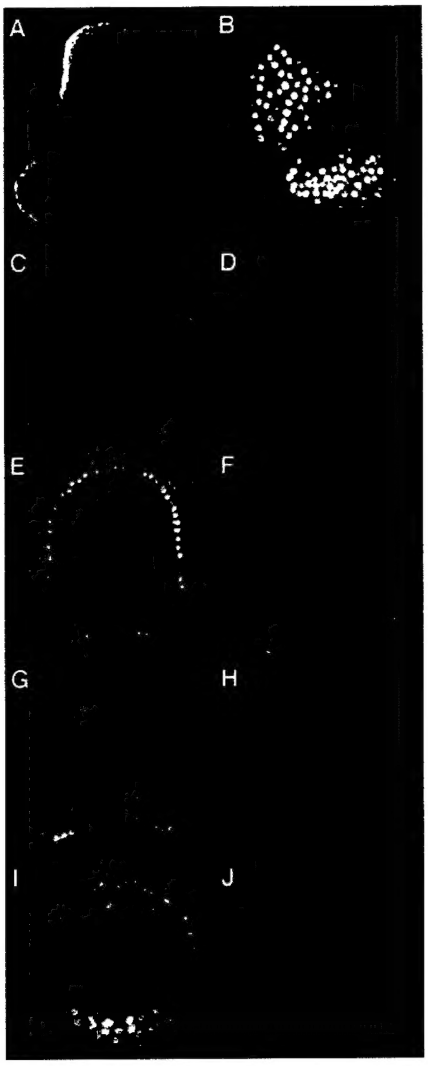

Fig. 7. Perturbation of the oral/aboral axis and the consequences for LvTbx2/3 expression. (A,B) Control embryos depicting normal expression of EctoV and LvBrac. (A) Cross-sectional view of EctoV oral ectoderm distribution in the late gastrula. (B) Mid-gastrula surface view of normal blastopore and somodael LvBrac expression. (C,D) Injection of *ΔLvG-cadherin* mRNA animalizes the embryo by binding to endogenous β-catenin and preventing its nuclear localization. These embryos lack endoderm and mesoderm and, as previously reported, express the EctoV antigen uniformly (C). They do not express aboral LvTbx2/3 (D). (E,F) $NiCl_2$ ventralization. (E) LvBrac expression expands to all ectoderm cells after ventralization with $NiCl_2$. (F) LvTbx2/3 is not expressed in any germ layer of these embryos. (G,H) Ectopic expression of *BMP2/4* radializes the ectoderm of the embryo, as indicated by the formation of multiple triradiate spicules (Angerer et al., 2000). Such embryos express normal levels of vegetal LvBrac around the blastopore but do not express oral LvBrac (G) and aboral LvTbx2/3 in the tissue of any germ layer (H), indicating that ectopic expression of *BMP2/4* antagonizes normal specification events along the A/V axis and in the O/A axis in all germ layers. (I,J) Disruption of the extracellular matrix with βAPN, a drug that prevents collagen crosslinking, results in the failure to express oral LvBrac, but vegetal expression of LvBrac is, apparently, unaffected (I). (J) βAPN also prevents LvTbx2/3 expression.

disruption is at the level of patterning and not at the level of specification appears to be conservative.

At 48 hours post-injection, the skeletons of embryos injected with *LvTbx2/3* lacked a consistent pattern, with each embryo elaborating a different, abnormal skeletal phenotype. Two such embryos are presented in Fig. 8, and it is clear that, when compared to a normal pluteus-stage embryo (Fig. 8A,B), patterning of the skeletogenic mesoderm was grossly perturbed (Fig. 8E-H). Embryos that expressed *LvTbx2/3* ectopically also had severe endodermal defects. In a few cases, exogastrulae were observed following ectopic *LvTbx2/3* expression (data not shown) but, most often, defects were manifest in an archenteron that had multiple 'chambers' rather than a typical tripartite structure. Despite their abnormal morphology embryos stained positively for the Endo1 antigen (5c7), which is normally expressed in the midgut and hindgut (Fig. 8I). Vegetal (blastopore) *LvBrac* expression in embryos that ectopically express *LvTbx2/3* was also normal, indicating that the endodermal defect was independent of LvBrac in the vegetal plate. In other words, it occurred after gastrulation (Fig. 8J).

It is well established that, in the sea urchin embryo, the skeletogenic mesoderm uses spatial and temporal patterning cues that are localized to the ectoderm to form appropriate skeletal structures (reviewed by McClay, 1999). The morphological skeletal abnormalities observed in embryos that express *LvTbx2/3* ectopically could result from inappropriate expression of either oral-specific or aboral-specific genes in the ectoderm that are induced by ectopic *LvTbx2/3* expression. Thus, downstream patterning cues would also be misexpressed or absent. Embryos were stained either 24 hours (data not shown) or 48 hours after ectopic expression of *LvTbx2/3* using antibodies against the two markers of oral ectoderm, EctoV and LvBrac (Fig. 8K,L). EctoV expression was confined to one region of the embryo, which indicates that the ectoderm contained an oral territory (Fig. 8K). LvBrac was expressed in a stomodael domain, indicating that substructures in the oral ectoderm were also specified (Fig. 8L). mAb 295 is an antibody that recognizes the ciliary band, a neurogenic region composed of both oral and aboral cells (Cameron et al., 1989). In embryos injected with *LvTbx2/3*, mAb 295 stained an amorphous region around the embryo, indicating that although oral and aboral territories have been specified and subdivided in the ectoderm, the boundary is not tightly localized (Fig. 8M).

DISCUSSION

LvTbx2/3: A T-box family transcription factor distributed asymmetrically in derivatives of all three embryonic germ layers

Here, we report the identification and characterization of a novel sea urchin T-box gene, an ortholog belonging to the *Tbx2/3* subfamily. The spatial restriction of LvTbx2/3 protein to the aboral regions of each germ layer demonstrates that there is polarized gene expression about the O/A axis in the ectoderm, endoderm and mesoderm of the sea urchin embryo. Because all three tissues share this molecular component, O/A specification in each does not involve totally unique sets of proteins. Perturbations of either molecules or pathways involved in O/A axis formation indicate that LvTbx2/3 acts

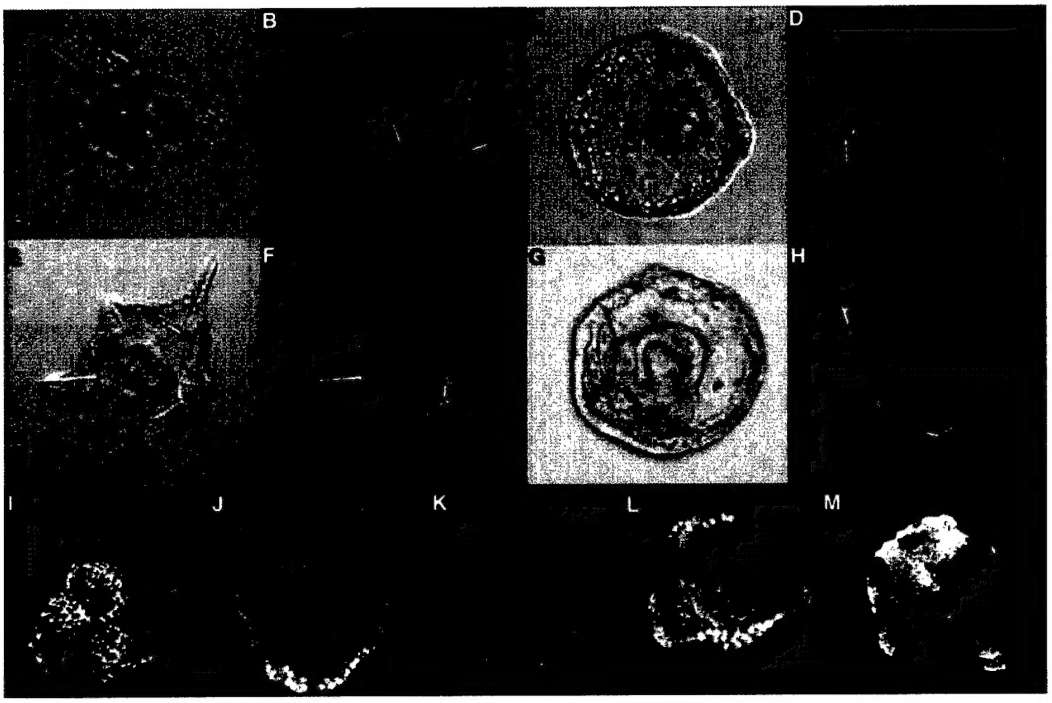

Fig. 8. Ectopic expression of *LvTbx2/3* mRNA causes profound morphological defects in embryonic development but does not prevent expression of markers of ectoderm, mesoderm and endoderm specification. Nomarski (A) and polarized light (B) images of 24 hour control, glycerol-injected embryos. These embryos exhibit the morphology, skeletal pattern and tripartite gut characteristic of the pluteus stage of development. Embryos that ectopically express *LvTbx2/3* mRNA (0.75-1.0 pg/pl; three to five times the endogenous copy number per nucleus, but in all nuclei) and imaged under Nomarski (C,E,G) or polarized light (D,F,H) optics are positioned to show a vegetal view. Twenty-four hours after injection, ectopic *LvTbx2/3*-expressing embryos appear radialized with multiple spicule clusters forming around the circumference of the embryo (D). They are, in many cases, delayed in gastrulation compared with control embryos as the archenteron has not yet reached the animal pole. The embryos contain derivatives of all germ layers, including pigment and blastocoelar cells derived from the nonskeletogenic mesenchyme, indicating that early specification events have not been eliminated. (E-H) embryos ectopically expressing *LvTbx2/3* for 48 hours. These embryos exhibit severe morphological defects in tissues derived from all three germ layers. They lack the typical pluteus form, have drastically mispatterned skeletons and have archenterons composed of multiple chambers rather than the normal three. Two embryos (F,H) display the variability in the skeletal phenotypes. No two embryos that ectopically express *LvTbx2/3* display identical defects in their skeletons, although all are severely mispatterned. When stained for terminal markers of pattern formation in these tissues, these embryos express markers for each known cell lineage. In the endoderm, the mid/hindgut marker, Endo1, is expressed and, in many cases, is localized to several of the additional chambers that have formed (I). LvBrac is normally expressed in two domains, a blastopore/hindgut domain and an oral/stomodael domain. Within the endoderm of injected embryos, LvBrac expression remains around the blastopore (J). EctoV is normally expressed in a refined domain corresponding to the oral ectoderm. In injected embryos, EctoV expression is still refined, indicating an oral axis has formed (K). (L) LvBrac is also expressed normally in the stomodael domain, indicating that substructures have been specified in the oral ectoderm and that domain is not 'aboralized'. (M) mAb 295, which recognizes the ciliated band, a structure at the boundary between oral and aboral cells, is also expressed in these embryos. However, instead of being a tight band, in many cases the ciliary band is broadly dispersed, indicating a loss of a refined O/A boundary.

downstream of these events and may be proximal to the morphogenetic events that shape aboral structures. To our knowledge, no member of the T-box gene family, or of any transcription factor family, has been described that is distributed in such a strikingly polarized manner in the three germ layers of any organism.

O/A polarity in the sea urchin embryo

That *LvTbx2/3* is expressed in the aboral territories of the endoderm, ectoderm and mesoderm provides a point of entry to examine the regulation of gene expression along the O/A axis in each of these tissues. To this end, we perturbed several events that are thought to be involved in patterning this axis and examined the effects on the expression of LvTbx2/3 and LvBrac, markers of aboral and oral gene expression, respectively. Results of these experiments suggest that either β-catenin or the expression of genes downstream of β-catenin is necessary for the expression of both proteins and, thus, gene expression along both the A/V and the O/A axes of the sea urchin embryo. Pharmacologically blocking formation of the aboral axis with $NiCl_2$ prevented LvTbx2/3 expression in all tissues, suggesting that gene expression along this axis is uniformly sensitive to this perturbation. In addition, it is possible that gene expression of axial information is controlled by mechanisms common to each germ layer rather than through different pathways in each.

The results on protein distribution after ectopic expression of *BMP2/4* are particularly interesting because previous experiments in *Strongelocentrotus purpuratus* embryos demonstrated that oral expression of EctoV was blocked by ectopic expression of *BMP2/4* but the aboral domain of Spec1 expression increased (Angerer et al., 2000). In our study, ectopic expression of *BMP2/4* in *L. variegatus* prevented the expression of both aboral LvTbx2/3 and oral LvBrac (Fig. 7G,H). Thus, two aboral genes, *Spec1* and *LvTbx2/3* differ in their response to ectopic expression *of BMP2/4*. The ectoderm of these embryos may be a locked in some sort of pre-aboral ectoderm state in which some aboral proteins are expressed but others are not because the signals necessary for their expression are inhibited by increased concentrations of BMP2/4. The most obvious explanation is that BMP2/4 might be a component of aboral specification, but, given the different *Spec1* and *LvTbx2/3* responses to *BMP2/4* perturbation, other, aboral-specification mechanisms must also exist. Evidence for a veg1-derived signal to overlying animal tissues has been observed recently (D.R.M. and J.M.G., unpublished observations), and this signal is sensitive to ectopic BMP2/4 expression. Thus, the defects in O/A gene expression described here might result from a perturbation to this signal. Further characterization of the BMP2/4 pathway, and the identification of more markers for O/A-axis formation in the ectoderm will likely clarify this issue. It is also possible that species-specific differences in specification of the oral and aboral axes might explain this discrepancy. *S. purpuratus* embryos at least partially differentiate aboral ectoderm autonomously, whereas *L. pictus* embryos require vegetal signaling to do so (Wikramanayake et al., 1995). Therefore, the loss of *LvTbx2/3* expression in embryos of *L. variegatus* following overexpression of *BMP2/4* could reflect a slightly different role of this pathway in specifying structures along the O/A axis in *Lytechinus* species of urchins than that in *S. purpuratus*.

LvTbx2/3 patterning and morphogenesis in the sea urchin embryo

Based on perturbation studies, *LvTbx2/3* expression is downstream of the specification of endoderm, mesoderm and ectoderm, including initial O/A specification events in these tissues. When ectopically expressed, *LvTbx2/3* consistently produces abnormal morphological phenotypes and patterning deficiencies in derivatives of each tissue. Nevertheless, markers for specific germ layers and axial regions are expressed (Fig. 8). Thus, what is the role of *LvTbx2/3* in the aboral territories? Genes downstream of LvTbx2/3 may be involved directly in patterning and morphogenesis, as suggested by the skeletal and endodermal phenotypes that result from the ectopic *LvTbx2/3* expression studies presented here. Several other T-box genes have also been noted to have distinct functions during morphogenesis. These include *Brachyury* in gastrulation movements (Kimmel et al., 1989; Conlon et al., 1996; Wilson and Beddington 1997; Gross and McClay, 2001), *Eomesodermin* in the formation of bottle cells and initiation of gastrulation (Ryan et al., 1996; Russ et al., 2000), *spadetail* in paraxial mesoderm migration (Griffin et al., 1998; Yamamoto et al., 1998), and *Tbx24* in somite segmentation (Nikaido et al., 2002). It will be of great interest to refine the position of *LvTbx2/3* in a network of O/A specification when more genes are identified in this gene-regulatory network. Also, using the differential screening technologies that are available currently, it should be possible to identify downstream targets of LvTbx2/3 and determine their roles in patterning and morphogenesis along this axis.

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